Universidade de Lisboa

Faculdade de Farmácia

PRODUCTION OF GLYCOLIPIDS USING A CELL-BASED APPROACH TOWARDS A POTENTIAL CANCER THERAPY

João Pedro do Carmo Costa

Dissertation Report supervised by Professora Doutora Maria Henriques Lourenço Ribeiro and co-supervised by Professora Doutora Isabel Alexandra Ribeiro Monge da Silva

Biopharmaceutical Sciences Master Degree

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Resumo

Tendo em conta as terapias oncológicas que neste momento estão disponíveis no mercado, é do conhecimento comum que é necessário investir e investigar o desenvolvimento de novos compostos que possam eventualmente ajudar as terapias convencionais ou até mesmo a novas, e por isso, este trabalho consistiu na produção de novos compostos por via biotecnológica. Nesta perspetiva, este trabalho focou-se na produção e otimização de glicolípidos, em especial trealose lípidos, com recurso ao *Rhodococcus erythropolis* e em testar a sua atividade citotóxica em células cancerígenas. A produção foi otimizada às 24 horas, de forma a ter uma maior rentabilidade de produto, a extração do mesmo foi feita com base em extração líquido-líquido, utilizando solventes orgânicos consistido em acetato de etilo e metanol, numa proporção de 8:1. Após secagem, este foi recolhido, solubilizado em dimetilsulfóxido (1% v/v na concentração final) e em tampão fosfato (~10% v/v, concentração final) após vários testes de solubilidade, devido à baixa solubilidade do trealose lípido. Este foi testado através do ensaio Cristal-Violeta, feito em células U87 – glioblastoma primário. Foi possível detetar atividade citotóxica nas concentrações elevadas, nomeadamente a 88 µg/mL, dando aso à possível continuação de estudos por forma a confirmar que de facto, estas substâncias produzidas naturalmente, têm atividade anticancerígena, podendo assim servir para uma abertura de portas para novas terapias oncológicas.

Abstract

Having in mind the current oncologic therapies that are in the medical field, it is known that it is necessary to investigate and invest in new therapies that can in the future help the traditional therapies or even substitute them further in the future, and so, that's why this work consisted in a the attempt to produce new compounds through biotechnology. In this perspective, this work focused on the production and optimization of glycolipids, especially trehalose lipids and testing its cytotoxic effects in a cancer cell line. The compound is a glycolipid, called trehalose lipid, which is produced in a form of congener of various trehalose lipids with different carbon chains. This compound was produced by *Rhodococcus erythropolis*. The production was optimized at 24-hour growth, in order to increase the productivity of the glycolipids. The extraction was done through liquid-liquid extraction by using a solvent based in methanol. After drying the solvent, the compound was harvested and solubilized in DMSO (dimethyl sulfoxide) and PBS (phosphate buffered saline) after various solubility tests, as this glycolipid was hardly soluble. The compound was tested with the Crystal-Violet assay, with U87 cells which are a primary

human glioblastoma cell line. It was possible to see a cytotoxicity effect at the highest concentrations, opening the possibility of continuing this work in the future, showing that it is possible to have new therapeutics against cancer, by using this naturally produced biocompounds.

Agradecimentos

Primeiramente gostaria de agradecer à minha família, namorada e amigos próximos por todo o apoio incondicional que demonstraram em toda esta jornada que foi a vida académica. Queria deixar também um agradecimento aos meus colegas de trabalho, nomeadamente à minha chefia empresarial, pela liberdade dada por forma a conseguir acabar a tese ainda que a trabalhar.

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List of Abbreviations

- $CaCO_3$ – Calcium carbonate;
- CMC – Critical Micelle Concentration;
- DMEM – Dubelcco's Modified Eagle's Medium;
- DMSO – Dimethylsulfoxide;
- DNS – Dinitrosalicylic acid;
- FBS – Fetal Bovine Serum;
- FT-IR – Fourier-transform infrared spectroscopy;
- GPY – Glucose-Peptone-Yeast extract;
- HPLC – High Performance Liquid Chromatography;
- MTP – Microplates;
- OD – Optical Density;
- PBS – Phosphate Buffer Saline;
- SF – Shake Flasks;
- SL – Sophorolipids;
- TDM – Trehalose dimycolates;
- TL – Trehalose lipids;
- TLC – Thin-layer Chromatography;
- TPS – Trehalose-6-phosphate synthetase;
- YM – Yeast extract-Malt extract.

1 - Introduction

Glycolipids are composed of a hydrophilic polar sugar head group (backbone) and a hydrophilic apolar lipid moiety anchoring the molecule in the membrane.[1] They are a structurally very heterogeneous group of cellular membrane-bound compounds found in all living cells from prokaryotic to eukaryotic cells, from bacteria to human. Glycolipids are essential constituents of cellular membranes and have an enormous number of functions. They are important for cell aggregation or dissociation, they may act as receptors and provide specific contact and they may deliver signals or receive them. Many glycolipids may modulate the immune response, for example, gangliosides and bacterial lipopolysaccharides (LPS).[1] Glycolipids are glycosyl derivatives of lipids such as acylglycerols, ceramides and prenols. Glycolipids can often work as biosurfactants, which are usually extracellular after being produced on living surfaces. These are amphiphilic compounds with lower surface tensions that have multiple applications in a wide range of industries, namely in plant protection in the form of microbial glycolipids which were efficient for the bio-control of pests such as arachnids, eggs, larva, grasshoppers and box-elder bugs, or in the food industry, with functions such as controlling the agglomeration of fat globules, stabilizing aerated systems, improving the shelf-life of starch-containing products, modifying rheological properties of wheat dough and improving the consistency and texture of fat-based products.[2] One of these glycolipids that work as biosurfactants are the sophorose and trehalose families.

Sophorolipids constitute one of the most promising glycolipid biosurfactants that are produced in large quantity by non-pathogenic yeast species such as *Starmerella bombicola* and *Rhodoturola bogoriensis*. These compounds have characteristics which are similar or even superior to other biosurfactants. High selectivity and specific activity in a broad range of temperature, pH and salinity conditions contribute for their usage in a different range of areas such as bioremediation, biomedicine, agriculture and others.
These are produced as a mix of structurally related molecules, reaching up to 40 different types and associated isomers which make their properties and applications vary depending on the composition of the mix. Lactonic forms are more hydrophobic whilst acidic forms are more hydrophilic. [3]
Produced extracellularly, these compounds can be structural components of cell membrane and can play an important role in cellular processes such as cell-cell interaction, cell signal transduction, cell proliferation and cell recognition. [4]

Sophorolipids group is also one of the most studied and promising glycolipids has they have high production yields and easy recovery processes. [5] They were initially discovered in 1961 [6], but for being environmentally friendly surfactants and for presenting different biological activities, the interest in these compounds as multifunctional materials has been attracting attention in diverse fields.

Amongst the different biological activities, they present high bactericide, fungicide, virucide, anticancer and anti-inflammatory effects. [7]

They also have biocidal properties, as they can be used for phytopathogens control as adjuvant in formulation of herbicides (surfactant properties). Sophorolipids are also very effective in inhibition of mycelial growth, motility as well as in the lysis of zoospore. Along with the low toxicity and biodegradability, the antifungal properties suggest that sophorolipids possess great potential as promising and environment-compatible bio control agent. [3]

Trehalose lipids are a group of glycolipids produced by several bacteria such as Actinomycetales, yeasts and fungus. They are among the best known biosurfactants, distinguished from rhamnolipids and sophorolipids in both composition and activity. [8] They were first described in 1933 in a study about chemical composition of the lipids of tubercle bacilli. [9] Structurally, trehalolipids consist in a hydrophilic moiety (trehalose) formed by two glucose units linked through the α,α-1,1-glycosidic linkage and a hydrophobic moiety represented by chains of fatty acids, such as succinic, octanoic, decanoic, and mycolic acids. The bonding makes trehalose very resistant to acid hydrolysis, and therefore is stable in solution at high temperatures, even under acidic conditions. Trehalose is the carbohydrate group of cell wall glycolipids in Mycobacteria and Corynebacteria. [10]

Trehalose lipids have different applications, such as in the environment, in the industry and in the biomedical area.

In many cases, environmental contamination caused by industrial activities is due to hydrophobic organic compounds and, what makes this difficult for remediation is because they easily bind to soil particles, making them less soluble and less bioavailable to microorganisms that can potentially degrade them. For instance, the application of trehalose lipids in such soils generally shows good results, enhancing the solubilization of these compounds as well as the biodegradation time. [11]

In industries, trehalose lipids can be used as a surface-tension lowering agent, for example, in oil refineries, where the oil recovery is poor due to either low permeability of the rocks forming the reservoir or alternatively because of the high viscosity of the crude oil, trehalose lipids can enhance this oil recovery, by lowering the interfacial tension

between the oil and the water.[11] It can also be used as a de-emulsifying stimulant, by breaking emulsions which forms at several steps of oil extraction and processing. [11]
In the biomedical area, since biosurfactants in general are safer than synthetic pharmaceuticals due to their biological origin, they could be used in cosmetic preparations, since trehalose lipids are less irritating than sodium dodecyl sulphate. [11]

The most important factor limiting the use of these biosurfactants, such as trehalose lipids, as an alternative to synthetic compounds is the high cost of production and downstream processing. However, in pharmaceutical and biomedical sectors that could prove beneficial since only a small amount of product is required. [11]

2 - Background

Sophorolipids consist of the glucose dimmer, sophorose, linked through a glycosidic bond to a hydroxy fatty acid. Sophorolipids are produced as a mixture of congeners that, individually, show variations concerning the acetylation grade of sophorose, the hydroxylation position in their chain, the saturation grade of their bonds and the chain length of the hydroxyl fatty acid. Apart from all those variations, they can also be present in an acidic or lactonized form. [12]

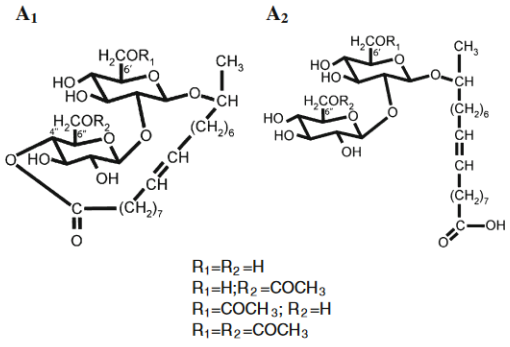

Figure 1 - Lactonic sophorolipids (A1) and acidic sophorolipids (A2) [30]

This glycolipid is one of the most promising and attractive bio-surfactants as it has numerous properties that make them superior to synthetic surfactants including: stability in the wide range of pH, temperatures and salinity, low-foaming and excellent detergent properties and are also readily biodegradable. [3] Apart from that, they also have further potential and applications as they act as biologically active compounds in biomedicine, particularly as anti-microbial, antitumor, antiviral and immune-modulator. Most of these

biological activities are probably because sophorolipids have the ability to destabilize cell membranes by changing their structure, cell permeability and these types of modifications will eventually lead to cell lysis.

Sophorolopids also displayed anticancer activity against several kinds of tumoral cells and might be of a potential use for cancer treatment. Sophorolipids synthesized by *Wickerhamiella domercqiae* have shown cytotoxic effects in several cancerous cell lines – they exhibited significant inhibition of cell proliferation on H7402 liver cancer cell line. This is due to the ability of these molecules to induce apoptosis. [3]

Trehalose is a nonreducing disaccharide with high thermostability, very resistant to acid hydrolysis, nonreactive to Maillard reaction and, due to its properties, has been considered an attractive compound for industrial applications, such as bioremediation as they reduce interfacial tension and are less sensitive to salt concentrations than synthesized surfactants. [10] This molecule is also associated with biological properties such as cryoprotection, growth regulation in plants, osmoregulation, suppression of "senior" body odor and protection of the cornea against oxidative damage caused by UVB Rays, acting as a suppressor of the proinflammatory cytokines. [13] It has also been described that trehalose has preventive effects against hepatic steatosis as it inhibits solute carrier 2A, member of a glucose transporter family. [14] Free trehalose disaccharides also act in the protection of biomolecules and cells against environmental stresses (heat, cold, oxidation and others). Based on this, it was proved that trehalose lipids are determining factors in protection of membranes, since its interaction with surrounding molecules can be considered essential to the development of resistant against desiccation by pathogenic *Mycobacterium tuberculosis.* [15] The biological activities of trehalose lipids include antiviral properties, inhibitory activity on calcium-dependent protein kinase C of human promyelocytic leukemia HL60 cells, inhibitory effects in grow and differentiation-induced against human leukemia cells and immunomodulation activity. [16,17,18,19,20,21,22] Also, just like sophorolipids, there is too the possibility of chemically modifying the glycolipid, obtaining novel analogues with diverse properties, and, it has been reported that the production of trehalose surfactants can be applied in medical field. [23] This relies on a construction of a liposome with DPMC + a 14-carbon derivative of a D-glycopyranosyl trehalolipid, this liposome proved to have apoptotic effects on HCT116 cancer cells as well as in MKN45 (colon carcinoma and gastric carcinoma cell lines). It was also proven that trehalose liposomes inhibit the growth of MOLT-4 cells in a dose-dependent manner (lymphoblastic leukemia cell lines)

and this was also studied in xenograft mice model. [24] In 2011, Gein et al. [25] proved that emulsions containing various concentrations of trehalose increased the TNF-α, IL-1β and IL-6 production when applied to adherent human peripheral blood monocyte cultures, suggesting that trehalose lipid has a lot of potential as an immunomodulatory and antitumor agent.

Derivatives of succinoyl-trehalose lipids can also induce differentiation activity in cells, with a specific interaction instead of a simple detergent-like effect. [26] It was also demonstrated that a derivative of trehalose, a 2,3-diacyl-D-trehalose-2'-sulfate can induce T-cells activity, in patients with tuberculosis, demonstrating the potential use of this compound in the development of new vaccines. [26]

2.1 - Biosynthesis

Sophorolipids pathway includes data collected from observations with different producing species, if the production process is equal or similar among every producing microorganism. The biosynthetic pathway can start from a fatty acid supplement of the fermentation medium or from the fatty acid synthesis. Fatty acid synthesis can occur from n-alkanes, alcohols, aldehydes or trilycerides supplementation to the media. [5]

Initially, from the action of NADP+, the fatty acid will be converted into a hydroxylated fatty acid. [5] Later, a mechanism firstly proposed for *Rhodotorula bogoriensis* was later confirmed for *Candida bombicola*, with a specific glycosyltransferase I will mediate the linkage between glucose at C1' position with hydroxyl group of hydroxyl fatty acid. Afterwards, a second transferase, glycosyltransferase II will link a second glucose molecule at C1'' position to the first C2' position. For the accomplishment of these linkages, a UDP-glucose is needed as a glycosyl donor for both transferases' activity. [27] This way a non-acetylated acidic sophorolipid is formed. This sophorolipid can also become lactonized by internal esterification of one hydroxyl group, which is thought to happen through the action of specific lipases or esterase that until now have not been identified. [28]

In terms of biosynthetic pathways for trehalose lipids, 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

alkanotrophic *Rhodococci*, TPS is induced by n-alkanes. [16] The further reactions involved in the synthesis of trehalose lipids have been clearly elucidated for trehalose dimycolates (TDMs) in *M. tuberculosis* in which the production occurs 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. [11]

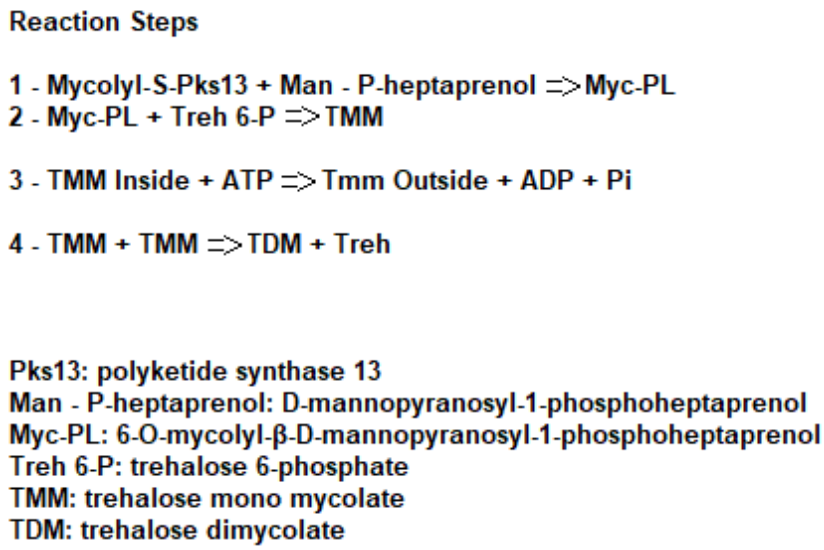

Figure 2 - Biosynthetic pathway of trehalose dimycolates. Steps 1 and 2 of the reaction are inside the cell, whilst step 3 represents the transfer to the outside. Step 4 occurs outside of the cell.

It is also known, that the carbohydrate and fatty acid moieties in the glycolipids of members of the genus *Rhodococcus*, are linked via an ester bond. The various biosynthetic pathways that are known directly depend on the carbon sources used for bacterial growth. For example, the use of n-alkane substrate will induce trehalose mycolates which are a predecessor from a trehalose-6-phosphate induction (caused by the n-alkane substrate). [23] Sugar substrates such as glucose, fructose or sucrose directly determine the carbohydrate moiety of the resulting sugar mycolates. [16]

2.2 - Production

When sophorolipids were firstly discovered in 1961 [6], was through the production of the yeast specie *Torulopsis magnolia*. Later, up until nowadays, this glycolipid production is being extensively studied through the production from *Starmerella bombicola* and *Rhodotorula bogoriensis* [29,30]. Sophorolipids are secondary metabolites, meaning that they are not essential for the yeast growth, development and reproduction. Even so, their

function is not entirely understood. They are produced extracellularly and, according to their biosurfactant potential, assumptions can be made that they can work as a biocide against other microorganisms, acting as a survival competitor mechanism. [31]

Different types of sophorolipid congeners can be obtained depending on which microorganism is used for their production and/or their growth medium. As an example, *Starmerella bombicola* is more likely to produce sophorolipids with a C18:0 chain, and mainly, with a C18:1 chain, in the lactonic form [29], whilst *Rhodotorula bogoriensis* will tend to produce more uncommon sophorolipids, with a lengthy chain of 22 carbons. [30]

Altogether, medium composition plays an important role in sophorolipids production, as it is not growth associated (secondary metabolites). From different carbon sources to different nitrogen sources as well as the usage of a lipidic co-substrate, it can directly influence the quantity of production, studies have shown [30]. The carbon substrate has a dual role in the fermentation process. It is consumed in order to assure energy generation and as a product for cellular biosynthesis. This works as a direct source of sophorolipid production but, with a lipidic co-substrate, it is possible to achieve higher yields. For example, batch production can be used in microtiter plates that work as a micro reactor, to test different substrates and calculate yield percentage production to later pass it into a reactor after studying the best growth and production conditions. There were already studies made with microtiter plates batch production as well as with fed batch production.

In terms of production, just like the other types of glycolipids, the microbial production of trehalose lipids can be affected by several factors such as carbon and nitrogen sources, salt composition and use of extract in culture broth. [32] Based on the published works, the yields of trehalose lipids production are very low when compared to sophorolipids, rhamnolipids and mannosylerythritol lipids. Among all tested trehalose lipid procures, only a study describes a high trehalose lipid yield, in this case using a *Rhodococcus sp.* SD-74 strain and achieving 40 g/L of the glycolipid using n-hexadecane under osmotic conditions. [33]

Trehalose lipids can also be produced with other microorganisms, such as *Tsukamurella spumae* or *Tsukamurella pseudospumae.* [34] Sunflower oil was used as a carbon source in 500 mL parallel bench-scale bioreactors. The process was run during 96 h and after measuring the crude trehalose lipid extract they were able to produce 1.28 g/L of total yield. [34]

Evaluating all the data that has been published so far, *Rhodococcus sp.* seem to be the most efficient bacteria producing these compounds. Espuny et al [35] used a mixture called P-120, which is composed of n-decane 9.4 %; n-undecane 32.2 %, n-dodecane 31%, n-tridecane 25.9 %, n-tetradecane 1.5 %. They also tried P-147, which is a mixture of n-tetradecane 33.6 %, n-pentadecane 56.6 %, n-hexadecane 8.5 %, n- heptadecane 1.3 %. Both were added at 2 % (v/v) in a 500 mL inoculum on shake flasks at 100 rpm. After testing the effect of the different carbon sources, the trehalose lipids yield was high using n-tridecane alone (2.43 g/L) than using P-147 (1.52 g/L). [35]

The main important points are the temperature, always at 30 ºC, the pH that should always be neutral and of course, the carbon source.

As stated above, using sugars as a carbon source directly affects the carbohydrate moiety, which might have a good effect in the trehalose lipid production and should also be cheaper. The bioreactor settings, the medium enriched with minerals and different big carbon chained substrates makes the large-scale production of trehalose lipids very challenging as it limits the effective use of biosurfactants due to the high cost of production and complex downstream processing.

2.3 - Downstream process

The downstream process is probably the biggest hurdle to surpass, as normally the purification of the target biological compound can account of over half of the production cost in many biotechnology applications. [31] This is obviously applied in the production of sophorolipids and trehalose lipids.

Since sophorolipids are also extracellullar products, there is an initial need to separate the supernatant from the biomass as well. Afterwards, a first extraction is usually performed with ethyl acetate. If it was used a lipidic co-substrate then a second extraction is usually necessary, with hexane, to remove the remaining co-substrate. Some authors also state that when produced in large quantities, sophorolipids can be obtained by decantation while according to others [33] when the oil is totally consumed, to heat the suspension until 60ºC and let it cool down at room temperate, will make sophorolipids to precipitate. If separation and purification of a specific congener is required, column chromatography separation or HPLC can be performed. [36] The separation methods are usually the same, by using the n-hexane extraction method or other compounds such as ethyl acetate or even chloroform to later precipitate together with methanol. The only

major difficulty in separating and purifying sophorolipids is that they range from the highly polar acidic forms to the relatively non-polar lactonic types. Several methods are available using chromatography, but each requires profiling of fractions by TLC to determine the separation of structural groups. [37]

The recovery process of trehalose lipids from *R. erythropolis* implies the usage of solvent extraction, which might represent a problem when trehalose lipids are applied into the biomedical area. This solvent extractions can be done through the choloform:methanol method (2:1), methyl tert butyl ether method (MTBE) or through a mixture of ethyl acetate:methanol (8:1). Just like in rhamnolipids, an acid precipitation can be used to isolate the glycolipids, this is advantageous since it removes protein co-isolated when glycolipids are obtained through organic extraction. An approximate determination of trehalose lipid content in a culture medium or an extract can be carried out in a similar manner to other glycolipids using the anthrone method. [38] This colorimetric assay works by reacting anthrone with the sugar part of the trehalose, forming a colored complex which can be quantified using a spectrophotometer. This is only a simple assay though as in the case of a proper quantification, HPLC methods should be applied.

For instance, electrospray ionization provides excellent glycolipid ionization when used for direct infusion or with HPLC-MS. Ionized molecules are detected by a mass analyzer according to their mass to charge ratio (m/z) and can be fragmented using collision-induced dissociation to provide valuable information about each structure and their isomers. [37]

Purification of trehalose lipids is generally carried out using either TLC (thin layer chromatography) or column chromatography. TLC has been extensively used to detect trehalose lipids in extracts while also providing information about the structural composition (although it is not the best method for structural characterization). Several solvent systems have been reported but the most extensively used system is chloroform:methanol:water (65:15:2 or 65:25:4). Using p-anisaldehyde, trehalose lipids will appear green, with monomycolates appearing near the point of origin whilst the dimycolates will present its mark slightly further. There is also a chance of getting other marks as other lipid components can also be detected, if on the extract. Also, noteworthy to mention, it is hard to do large scale purifications using column chromatography, as these molecules are generally produced at low concentrations and thus represent a minor component of the crude extract sample. [11] The presence of different structural

types of trehalolipids and many other lipid type materials along with excess n-alkane used as substrate in the production process complicates the purification process further. Consequently, a preliminary column chromatography step has been suggested to remove hydrocarbons before a subsequent column chromatography for the purification of trehalose lipids using chloroform:methanol mixtures. [39]

Chloroform:metanol mixtures are the most commonly used solvent system although MTBE (methyl tert butyl ether) is used too. Kuyukina et al [40] noted that differences in the amount of biosurfactant extracted by tested solvent systems were small, but the use of MTBE and choroform-methanol resulted in a greater crude extract than the others.

Another recent extraction process is using a ethyl acetate:methanol (8:1) solution [11], consisting in a liquid:liquid extraction through a separation of an organic and aqueous phase. This extraction process can also be carried out in a similar manner to rhamnolipids, by extracting three times the broth with equal volumes of choloform:methanol (2:1) and further removing the solvent through rotary evaporator. [41]

2.4 - Characterization

Quantification and characterization of the sophorolipids is usually done with thin layer chromatography as most of the studies presented so far do. A different approach can also be done by using HPLC of mass spectrometry. [5] This assay can also give us valuable information regarding structural analysis, since mass spectrometry allows individual separation and characterization of the congener's mixture, having in mind that sophorolipids are usually produced in a compound mixture.

Just like other glycolipids, structural characterization of trehalose lipids can be done using numerous techniques, either by breaking down their structure separating the fatty acids from the carbohydrate or just by analyzing the entire molecule. The main technique is mass spectrometry. Mass spectrometry can be done through Gas Chromatography (GS), where a preparation of the purified trehalose lipids is done through an alkaline hydrolysis, where the glycolipids are broken down and the lipid portion is converted into fatty acid methyl esters. This characterization is necessary as it provides essential information that is needed to identify their structure. In terms of the carbohydrate molecules, they are converted into trimethylsiliyl derivatives, providing then information about the ester linkages between the fatty acids and then trehalose. [42]

More recently, Patil et al. [43] used FT-IR for functional group determination. FT-IR was carried out and it managed to indicate the carboxylic acid esters of the -CO group of the sugar groups as well as alkane groups of long-chain fatty acids. With HPLC-MS they were able to reveal the glycoside linkage between two of the sugar moieties, and together with all that data they were able to conclude that they had extracted TDM's (trehalose dimycolates).

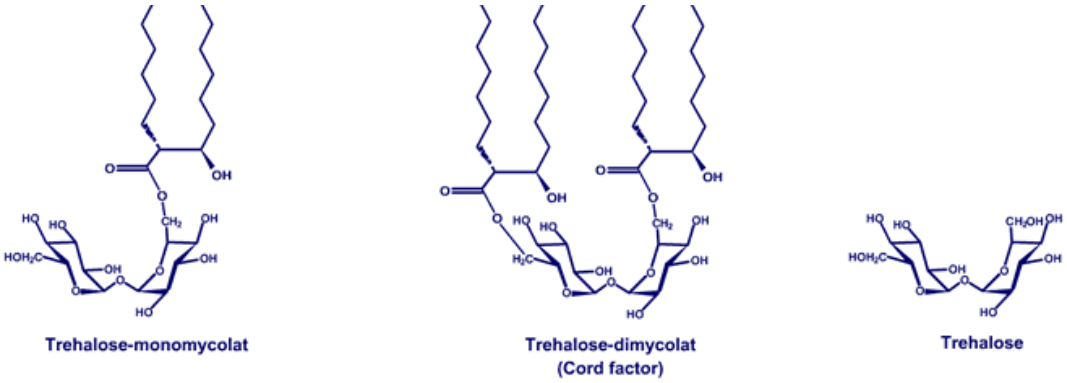

Figure 3 - Structural image of trehalose sugar and both of its most known glycolipid form, trehalose-monomycolate and trehalose-dimycolate (the later showing as an example, the cord factor, a known molecule from M. tubercolosis now being researched in a therapeutic matter, against cancer cells as it recruits IL-12 and IFN-y cytokines, that are able to limit the growth of tumors. [44] (Taken from blueTB pathways – FTP server with no copyrights)

2.5 - Surface Activity Relationship

Surfactants are surface-active compounds capable of reducing surface and interfacial tension at the interfaces between liquids, solids and gases, thereby allowing them to mix or disperse readily as emulsions in water or other liquids. The enormous market demand for surfactants is currently met by numerous synthetic, mainly petroleum-based, chemical surfactants. These compounds are usually toxic to the environment and non-biodegradable. They may bio-accumulate, and their production processes and by-products can be environmentally hazardous. Tightening environmental regulations and increasing awareness for the need to protect the ecosystem have effectively resulted in an increasing interest in biosurfactants as possible alternatives to chemical surfactants. [45]

The balance between the amphiphilic structure of the sophorolipids molecules plays an important role on their own surface properties. With that, acetylation grade, chain length of sophorolipids and sophorolipids configuration profile will directly influence critical micelle concentration (CMC) and, consequently, surface tension. Studies have shown that there is a difference in surface tension at CMC depending on the acetylation grade. For instance, it has been observed that a monoacetylated C18:1 lactonic sophorolipid has higher surface tension than a diacetylated one. Also, an important detail to note is that, surface properties of sophorolipids are dependent on the co-substrate supplemented to the fermentation media because it will directly influence their own congener mixture. For example, sophorolipids obtained from soy molasses/oleic acid fermentation lowers the surface tension of water to minimum surface-tension value of 36-38 mN/m. [46]

Surface tension and emulsifier index of trehalose lipids are one of the characteristics that gives them potential to have future applications in the industry. Because of their dual structure, surfactants tend to absorb the interface in an oriented fashion. Moreover, this structure is responsible for lowering the interfacial tension and increasing the miscibility of the liquids. In general, the presence of biosurfactants tends to decrease the interfacial tension to values close to 1 mN/m and, for this reason, they are very effective in promoting hydrocarbon emulsification and enhancing oil recovery. [42] They've also shown that depending on their structures, they can range from simple micelles to more complex liquid crystals (at Critical Micelle Concentration).

Most of the trehalose lipids that have been examined in the genus *Rhodococcus*, showed strong surface activity by lowering water surface below 30 mN/m, lowering the interfacial tension against hexadecane (decane or kerosene) 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. For instance, the glycolipid synthetized by the specie DSM43215 showed extremely low critical micelle concentration in high-salinity solutions with the interfacial properties staying stable in a wide range of pH and ionic strength solutions, showing great potential in being applied in an enhanced oil recovery system. [10]

2.6 - Other Applications

It is generally believed that biosurfactants are more environmentally friendly alternative to synthetic surfactants because of their lower toxicity and higher biodegradability. However, the toxicity of microbial produced surfactants should always be assessed, especially when an *in-situ* application is planned – because depending on the strain that produced such compounds and the extraction processes as well as the downstream process that was undertaken, might directly affect its level of pathogenicity.

Sophorolipids have several other applications, for instance, 1 % (v/v) of sophorolipids in a germicide formulation is enough to kill 100 % of *Escherichia coli*, *Salmonella typhi* e *Shigella dysenteriae* in 30 seconds after application of germicide. [41]

There are several other applications for trehalose lipids, for instance, Tomasz et al. [41] propose that they can be used as surface coating agents, because of their ability to inhibit microbial colonization of polystyrene and silicone surfaces – they demonstrated 95 and 70 % antiadhesive activity against *Candida albicans* and *Escherichia coli*, respectively.

Produced by *Rhodococcus* H13-A, trehalose lipids were more effective than a synthetic surfactant called Tween 80, in the enhancement of the aqueous concentrations of several Polycyclic Aromatic Hydrocarbons from crude oil. It also helps solubilize compounds such as dibenzothiophene, naphthalene or phenanthrene in water. [11]

3 - Objectives

The main objective of this thesis consists in the development of a fast and non-expensive way of producing glycolipids, such as sophorolipids and trehalose lipids, in order to investigate their possible cytotoxic effect against cancer cells.

4 - Materials and Methods

4.1 - Materials and reagents

For the experiments done, usual laboratory materials were needed in order to do all the incubation process, like cell growth and the obtaining of the supernatant and for the extraction and purification of the trehalose lipids, such as: microplates; erlenmeyers; micropipette tips; graduated pipettes; test tubes; eppendorfs; bunsen burners; petri dishes; volumetric flasks and watch glasses.

For the experiments done, the following reagents, all of them from Sigma-Aldrich, were used:

Table 1 - Reagents used and brand.

Reagents	Brand
Yeast extract	Sigma-Aldrich
Malt extract	Sigma-Aldrich
Tryptic Soy Broth	Sigma-Aldrich
Dulbecco's Modified Eagle's Medium	Sigma-Aldrich
Peptone	Merck
Glucose	Sigma-Aldrich
Calcium carbonate	Merck
3,5-dinitrosalicylic acid (DNS reagent)	Merck
Acidified solution of Coomassie G-250 (Bradford reagent);	Bio-Rad
Acetic acid	Merck
Sulfuric acid	Merck
Chloroform	Merck
Methanol	Merck
p-Anisaldehyde	Sigma-Aldrich
Ethyl acetate	Merck
Phosphate-buffered saline	Merck
Ethanol	Merck
Crystal violet	Sigma-Aldrich
Distilled water	Produced internally - MilliQ

4.2 - Equipment

As far as equipments are concerned, the following ones were used in order to do all the growths and assays needed to complete this work:

Table 2 - Equipments used and brands.

Equipment	Brand
Incubator	Aralab agitorb 160E
Bioreactor	Infors AG CH-4103
Peristaltic Pump	Gilson Minipulse 2
Laminar Flow Chamber	C.R.C. 5605
Water Bath	Memmert
Centrifuge	Biofuge Pico - Heraeus
Analytical Balance	A-150-SX - Cobos Precision
Microscope	Axioimager - Zeiss
Spectrophotometer	Zenyth 3100 - Anthos
Autoclave	AJC Amaro - 2000
Rotary Evaporator	IKA RV-10 - VWR
Biophotometer	Eppendorf

4.3 - Microorganisms, media and glycolipids production

4.3.1 - Sophorolipids

Starmerella bombicola was from CBS (Centraalbureau voor Schimmelcultures) Fungal Biodiversity Centre and was maintained on Glucose-Peptone-Yeast extract (GPY) agar slants.

For the preliminary assays for culture growth of *Starmerella bombicola* in the bioreactor (volume total of 3.2 L and a useful volume of 2 L), 100 mL of GPY seed medium (20 g/L glucose; 5 g/L yeast extract; 5 g/L peptone and 0.2 g/L oleic acid) was grown for 72 h at 30 ºC on an orbital shaker (150 rpm). Afterwards, it was inoculated in the bioreactor (Figure 3). The culture growth and sophorolipids (SL's) production conditions set in the bioreactor were the following: temperature 28 ºC; pH 7.0; stirring speed 200 rpm and dissolved oxygen 50 %.

The GPY medium (2 L with 1 mL of anti-foam) in the bioreactor vessel and all the accessories were sterilized (AJC Amaro 2000 autoclave). The samples were aseptically withdrawn. Cellular growth and SL's production were initiated, inoculating a seed medium volume equivalent to 5 % (v/v) of sterile fermentation medium (GPY).

Culture media samples were taken periodically, using a peristaltic pump, along 4 days from the vessel for biomass, glucose consumption and SL's production. Biomass estimation was followed at OD600 nm, protein quantification by Bradford method and glucose quantification by DNS method.

The SL's production was followed using thin layer chromatography. The mobile phase composition was chloroform: methanol: water (65:15:2) and the stationary phase was Merck silica Gel 60 F254 20x20 TLC plates. The visualization of the SL's bands was performed by spraying the TLC plate with p-anisaldehyde reagent and heating at 110 ºC for 10 min.

Figure 4 - Bioreactor used to produce sophorolipids.

4.3.2 - Trehalose lipids

Rhodococcus erythropolis, DSM 43066 from the German Resource Centre for Biological Material, DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH), was used to produce trehalose lipids. This microorganism is usually found in soil.

Rhodococcus erythropolis was acquired in a lyophilized state, it was then sub-cultured in TSB medium (DSMZ), in five vials with 5 mL of media each. They were grown at 30 ºC for 24 hours. After those 24 hours, four plates were cultured with TSB in agar

slants, for 24 hours as well. Afterwards, *Rhodococcus erythropolis* was maintained frozen at -80 ºC.

Rhodococcus erythropolis were maintained at 4 ºC, on Yeast extract–Malt extract (YM) agar slants, with monthly transfers.

Afterwards, growth and TL's production were carried out in YM Medium (yeast extract-malt extract medium), which is composed of 3 g/L of yeast extract, 3 g/L of malt extract, 10 g/L of glucose and 2.0 g/L of calcium carbonate ($CaCO_3$). A pre-culture was initially grown for 24h in an orbital shaker (150 rpm) at 30ºC.

The effect of inoculum concentration and calcium carbonate ($CaCO_3$) presence were studied in 24-wells microplates (MTP) and shake flasks (SF) from 24 to 96 hours. All assays were carried out in triplicates.

The different media used were sterilized at 121 ºC in an autoclave and aseptically prepared in a laminar flux chamber.

4.4 - Cells, media and cytotoxic assay

U87 cell line (American Type Culture Collection, ATCC® HTB-14™), which is a human primary glioblastoma cell line, was used to do the cytotoxicity tests. Cells were grown in Dubelcco's Modified Eagle´s Medium (DMEM), supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin/streptomycin solution, for 48 hours, at 37 ºC, in a CO_2 incubator with humidified atmosphere and 5 % of CO_2.

4.5 - Analytical Methods Development

4.5.1 - DNS Method

DNS (dinitrosalicylic acid) assay consists in the use of 3,5-dinitrosalicylic acid, an agent that reduces sugars, by transforming their carbonyl groups in carboxylic groups, turning the tested samples from orange color to a browner colored sample, through heat activation.

In a microplate, different wells were filled with 75 µL of DNS reagent plus 75 µL of the testing sample. Seven different glucose standard solutions were prepared beforehand (0.2 mg/mL; 0.3 mg/mL; 0.4 mg/mL; 0.5 mg/mL; 0.6 mg/mL; 0.7 mg/mL and 0.8 mg/mL) and the microplate was then put in a water bath at 95 ºC for 5 minutes, in order to obtain a calibration curve and to determine the respective equation (absorbance versus concentration), as shown in figure 3.

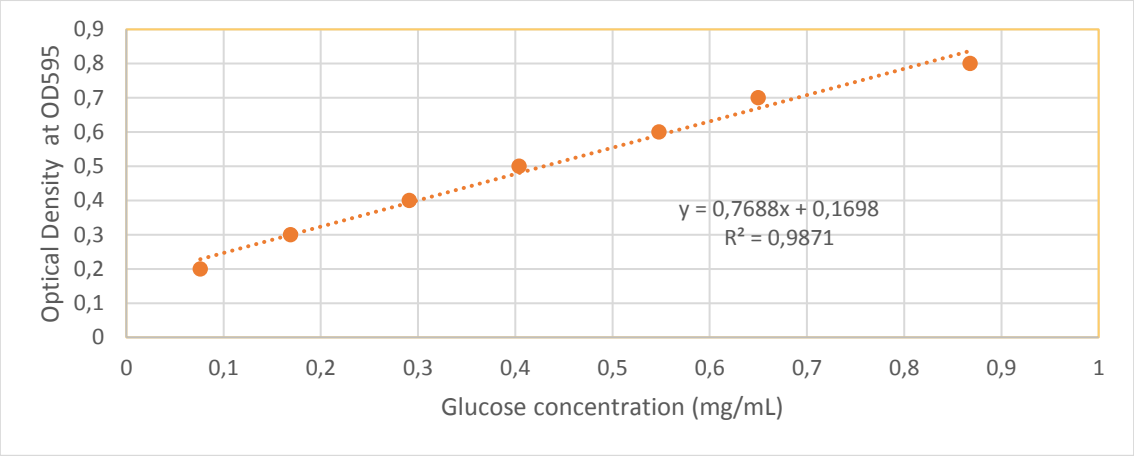

Figure 5 - Glucose calibration curve for the DNS method.

4.5.2 - Bradford Method

The Bradford (Coomassie blue G-250 dye-binding) assay is a common colorimetric assay used to determine the concentrations of proteins. The assay produces a colored solution in the visible spectrum in response to protein presence. The color formation observed in the Bradford assay is a result of interactions between the protein and the Coomassie blue G-250 dye. Through electrostatic and hydrophobic interactions with a protein molecule, the anionic (−1 net charge) blue form of the dye is stabilized. [47]

In a microplate, different wells were filled with 150 µL of the dye reagent plus 150 µL of the testing sample, albumin. Seven concentrations were tested, ranging from 0.125 mg/mL to 2 mg/mL, in order to determine the calibration curve in figure 4 (samples were analyzed in a biophotometer).

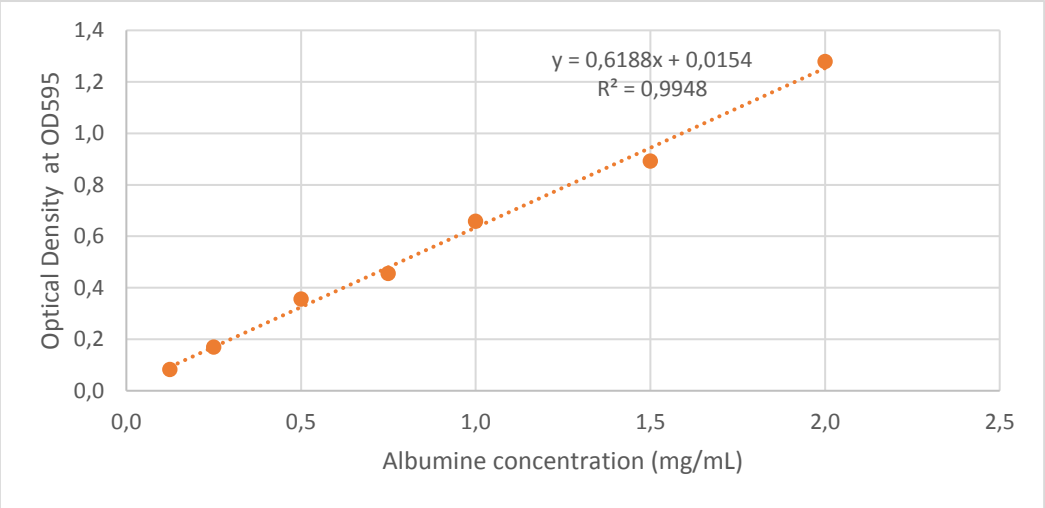

Figure 6 - Calibration curve for the Bradford test.

4.5.3 - Thin-layer Chromathography

In a first detection of the product and fermentation monitoring, thin layer chromatography (TLC) was carried out. Just like any chromatography, TLC has a stationary phase and a mobile phase. The mobile phase flows through the stationary phase and carries the components of the mixture with it. Different components travel at different rates and that's how the mixture is separated and identified. Using a thin, uniform layer of silica gel, with a fluorescent substance, the sample can be analyzed after using UV light. During TLC, the solvent began to soak up the plate, it first dissolved the compounds in the spot put on the base line. How fast the compounds get carried up the plate depends on how soluble the compound in the solvent is being used and how much the compound sticks to the stationary phase. In this case, 20 µL of the extract was put in a TLC plaque made of silica gel 60 F254 and the mobile phase was a mixture of chloroform-methanol-water solution (65:15:2). After 2 hours of saturation, the plaque was sprayed with p-anisaldehyde solution (100 mL of acetic acid + 2 mL of sulfuric acid + 1 mL of p-anisaldehyde) being then heated up to 105ºC until the stains started to appear.

4.5.4 - High Performance Liquid Chromatography Mass Spectrometry (HPLC-MS)

HPLC-MS is a powerful tool in analysis. It's basically a highly improved form of column chromatography as 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 much faster and allowing a much better separation of the components in the mixture. The time taken for the glycolipids to travel through the column is known as retention time and that is one way to separate the compounds because most of the retention times are already published in literature, making it easier to compare and confirm the presence of the compounds of interest. That retention time depends entirely on four factors, the pressure used, the nature of the stationary phase as the molecular interaction plays a key role, the composition of the solvent and the temperature of the column during the process. 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 (just like the retention time). [48]

The compositional analysis of trehalose lipids produced by the *Rhodococus erythropolis* was performed by LC–ESI-MS/MS. A reverse phase Waters™ Atlantis C18 column (150 mm×2.1 mm id,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 is presented in Table 1. The flow rate was constant at 0.3 mL/min, the injection volume was 4 µL and samples were maintained at 10°C. The equipment was controlled by Waters Empower Software. The instrument was operated in the positive ion electrospray mode, ESI capillary voltage was optimized to 4 kV, cone voltage was set on 60 V and source and desolvation temperature were adjusted to 120 and 350 °C, respectively. Data acquisition and mass spectrometric evaluation were performed with MassLynx Software 4.1.

The samples (200 µL) were solubilized in acetonitrile (ACN) (800 µL). However due the low solubility of the compounds they were not completely solubilized. The elution conditions (flux 0.3 mL/min) are shown in table 1.

Table 3 - HPLC conditions, using a column Sunfire C18, 5 µm, 100 x 2.1 mm with the eluents water and acetonitrile (ACN).

Time (min)	H_2O (mL)	ACN (mL)
0	50	50
2	50	50
7	5	95
12	5	95
20	50	50

4.5.6 - Separation and Purification of Trehalose lipids

The separation of TL´s from the growth media may present some difficulties; since they are blends of compounds with very similar properties. Liquid-liquid extraction was used, testing different mixtures of solvents in order to develop the best conditions for TL's purification. The first liquid-liquid extraction method tried was a 1:1 ethyl acetate:growth medium. The samples were centrifuged at 5000 rpm for 10 minutes but unfortunely it was not possible to separate the samples in an organic phase and aqueous phase.

So, just like Patil [43], an ethyl acetate methanol solution was used (8:1). The entire culture media (50 mL and 100 mL) was shaken vigorously for about 5 minutes until a very clear separation between the organic and the aqueous phase was seen. The TL's were in the organic phase, so the aqueous phase was discarded from the separating funnel and the organic phase was then collected. Afterwards, sodium sulphate anhydrous was added in small quantities to the organic samples, in order to dry out the water. After the solvent was evaporated from the samples in a rotary evaporator, the solid samples were then collected from the flask and later were weighted, in order to evaluate the yield of the bioprocess.

4.6 - Cytotoxicity Test – Crystal Violet Assay

U87 cells (0.4-0.6 x 10^5 cells/mL) were exposed to TL's (a range of concentrations from 0.225 µg/mL to 8.8 µg/mL with DMSO as solvent and 2.25 µg/mL to 88 µg/mL with PBS as solvent) during 24-hours and two independent 48-hours period, DMEM (Dulbecco's

Modified Eagle Medium) Medium was used. After the exposure time, the 96-well microplate was washed with PBS at 37 ºC. Cells were fixed with a 96 % ethanol solution (200 µL/well) for 10 minutes. After the fixation, the ethanol solution was removed and a water-based solution of crystal violet 0.1 % (v/v) + ethanol 10 % (v/v) was added for some minutes (2-3 minutes). The plate was then washed with current water, minimally dried and then each well is re-suspended with a 96 % ethanol + 1 % acetic acid (v/v) solution - absorbance was then read.

In this assay, the TL's were first solubilized in DMSO, the final solution (0.012 mg/mL) was diluted 1/10 with distillated water, so the DMSO concentration was about 1 %(v/v) as the U87 cell line is very sensitive to DMSO (the positive control used in this assay was DMSO 10 % (v/v)). Due to reduced results, the assay was rebuilt, by extending the treatment period to 48 hours and solubilizing TL's in phosphate buffered saline (PBS) – since PBS (~10% v/v) is not harmful to the cells a higher concentration of TL's was achieved (88 µg/mL).

Table 4 - Concentrations used in the two independent assays with a 48-hour period of exposure time, using solvents DMSO (1% v/v at final concentration) and PBS (~10% v/v at final concentration) for the solubilization of TL's.

	Solvent	
	DMSO	**PBS**
TL´s concentration (µg/mL)	8.8	88
	4.4	44
	2.2	22
	1.1	11
	0.55	5.5
	0.225	2.25

5 - Results and Discussion

One of the goals of this work consisted in the development of a viable and economic process to produce glycolipids, namely sophorolipids (SL's) and especially trehalose lipids (TL´s) produced by fermentation. The biosynthesis of the glycolipids is dependent on the microorganism and on the environmental conditions, therefore in this work, *Starmerella bombicola* was used for sophorolipids and *Rhodococcus erythropolis* for the trehalose lipids production, under different conditions.

5.1 - Sophorolipids production in a bioreactor in batch mode

In previous works, SL's were produced in microwell plates and in shake flasks and the growth conditions were optimized and compared [30]. Therefore, in this work the SLs production was attempted in a bioreactor (3.2 L) (figure 3) in batch mode, in order to improve the *Starmerella bombicola* growth and SL's production.

The growth was followed during four days at environmental controlled conditions, namely temperature, pH, antifoam and dissolved oxygen.

As it is observed in figure 6, after 48 hours the growth was almost constant (7.5 mg/mL of cells) and the glucose concentration decreased in the same proportion (0.6 mg/mL).

The kinetic parameters are presented in table 2. A maximum growth rate (μ_{max}) of 0.37 h^{-1} was observed. This value and the maximum *S. bombicola* concentration (8.1 mg/mL) were higher (almost two-fold) than which were attained in shake flasks using the same medium and similar operational conditions [30] (see 4.3.1). This difference may be due to the controlled conditions in which the cells have grown in the bioreactor, namely the pH and the dissolved oxygen. It is known the importance of dissolved oxygen in these bioprocesses, besides the substrate's concentrations and the environmental conditions.

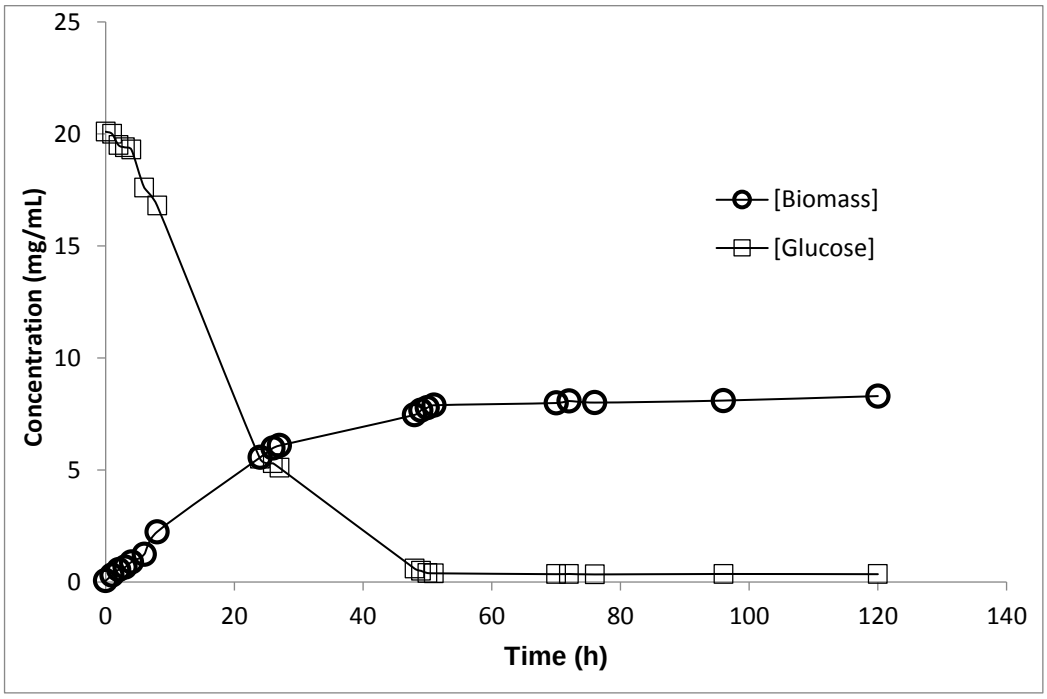

Figure 7 - Starmerella bombicola growth (open circles) and glucose consumption (square marks) in batch mode in a bioreactor, at 30 ºC, pH 7, oxygen dissolved 50 %.

Table 2 showed the kinetic parameters for *Starmerella bombicola* growth. A volumetric growth rate was observed, 0.27 g/L.h, while the glucose consumption rate was 0.41 g/L.h and 66 % yield.

Table 5 - Kinetic parameters for Starmerella bombicola growth and glucose consumption.

Kinetic parameters	
Maximum growth rate – μ_{max} (h^{-1})	0.37
Volumetric growth rate – r_x (g/L.h)	0.27
Glucose consumption rate – r_s (g/L.h)	0.41
Cellular yield – $Y_{(x/s)}$	0.66

The SL's production was evaluated by TLC. It was observed similar spots as in the reference [49] where a comparison of the TLC and HPLC-MS/MS were carried out. Based on that paper it was possible to conclude that the major sophorolipids produced were in the lactonic form.

In future work the production in fed batch mode should be evaluated based on the carbon source (e.g. glucose) and lipid source (e.g. oleic acid) additions, in order to continue the improvement of the SLs production and its selectivity.

5.2 - Trehalose lipids production

Cell growth and metabolites biosynthesis are strongly dependent on the composition of the growth medium including the carbon sources, the nitrogen sources, growth factors, and inorganic salts. In this work the production of trehalose lipids was developed in YM medium. This medium is strongly supported by glucose. Shake flasks and 24-wells microplates were studied as different strategies to economize and optimize the trehalose lipids production.

5.2.1 - *Rhodococcus erythropolis* growth in microplates vs shake flasks

One of the goals of this work was to compare *Rhodococcus erythropolis* growth in microplates and in shake flasks, in order to evaluate which had the best outcome in terms of growth and glucose consumption, which would lead to the production of TL's. A 24-hour growth was initiated through a pre-culture, using 5 % (v/v) of inoculum.

Bacteria growth conditions were compared in 24-wells microplates (MTP) and shake flask (SF), using 1 mL and 150 mL, respectively. The best results were attained in shake flasks (SF) (Figure 7). In fact, after the 24-hour incubation period, absorbances were read and these readings have shown that, it would be much better to use shake flasks going forward as we had much more bacterial growth in the flasks rather than in the microplate. This might be due to the fact that the bacteria surface area that is in contact with the medium in shake flasks is much bigger than the ones in the microplate, so during the growth, in the microplate, the bacteria might be limited to that area as it was grown more and more, so the duplication time was longer, all of those effects might also affect the production of TL's and so, moving forward, the bacterial growth was mainly done using shake flasks. Additionally, the headspace of SF allowed higher percentage of oxygen available for *Rhodococcus* growth.

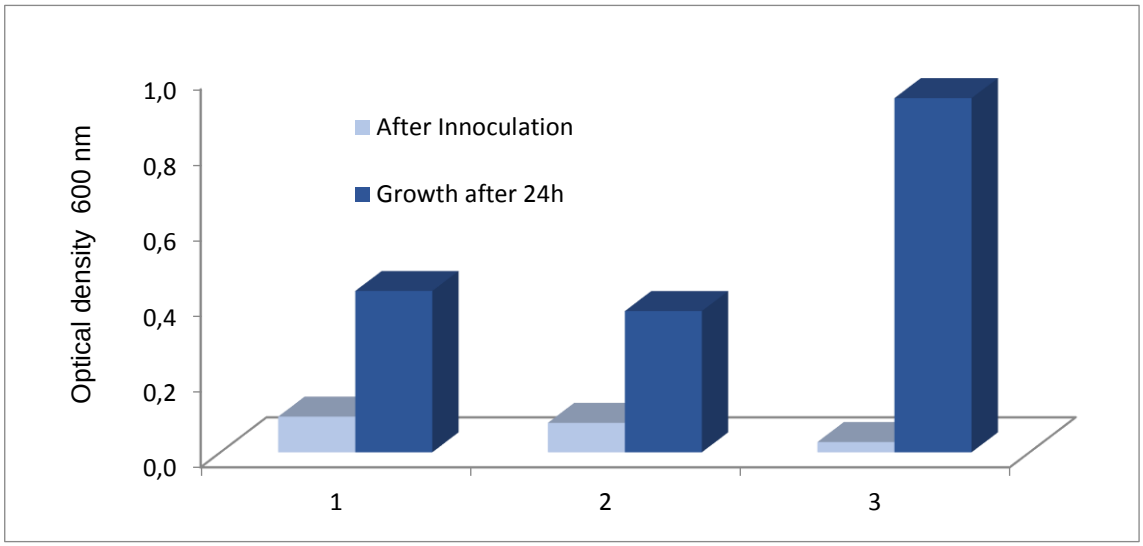

Figure 8 - Rhodococcus erythropolis growth. 1 and 2 represents MTP growth whilst 3 represents shake flask growth.

5.2.2 - Effect of $CaCO_3$ on *Rhodoccocus erythropolis* growth and TL's production

Initially, the effect of $CaCO_3$ was evaluated on *Rhodococcus erythropolis* growth and TL's production in 24-well microplates and in shake flasks. Initially $CaCO_3$ was studied in a concentration of 2 g/L (Figure 8).

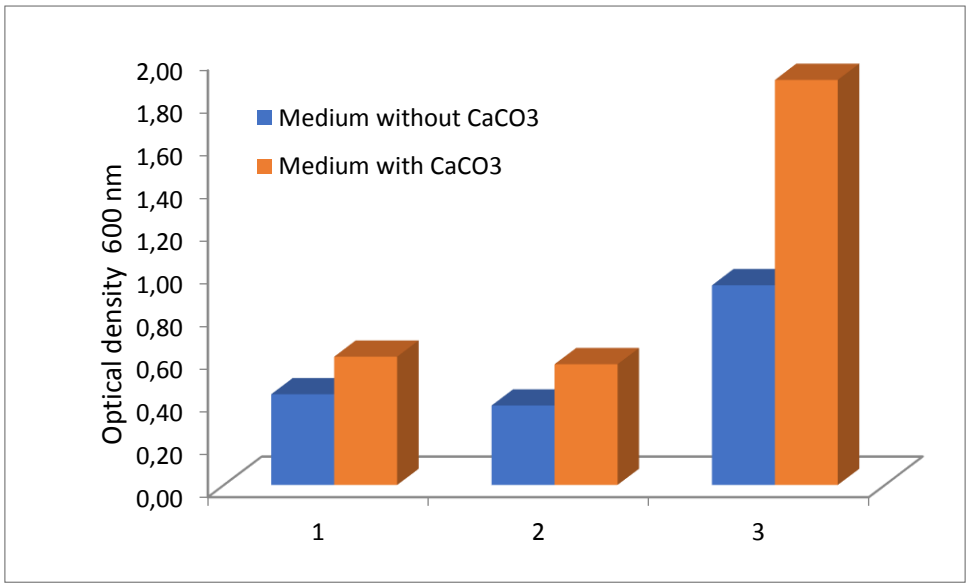

Figure 9 - The effect of $CaCO_3$ on Rhodococcus erythropolis growth. (Microplates 1 and 2 and shake flasks 3)

The best growth of *Rhodococcus erythropolis* was attained in the medium with $CaCO_3$. In fact, it was observed that $CaCO_3$ stimulates bacterial growth. Todescato et al [50] has also observed a positive effect of $CaCO_3$ on the growth of *Rhodococcus erythropolis* ATCC 4277. They evaluated the effect of $CaCO_3$ concentrations between 2.0 g/L and 1.16 g/L and the best growth rate was attained with the last concentration. [50]

Since $CaCO_3$ is hardly soluble it might have precipitated in the 24-well microplates (Figure 8), therefore physically blocking the bacteria from acquiring nutrients from the media. Moreover, shake flasks had a bigger area, even though the $CaCO_3$ also precipitated. Afterwards, it was decided to test different concentrations of calcium carbonate, since 2 g/L would be affecting the bacteria growth, and it was also precipitating in the medium.

Therefore, previously it was tested different $CaCO_3$ concentrations, to evaluate which solubilized completely in the in the YM media. The higher $CaCO_3$ concentration that allowed solubilization was 100 mg/L. So, *Rhodococcus erythropolis* growth was evaluated in shake flasks using this $CaCO_3$ concentration, at 30 ºC, 150 rpm, for 24 h. An optical density of 2.6 was observed, which corresponded to approximately 2.1×10^9 cells/mL.

The TL's production was evaluated by TLC. Similar spots were obtained in both conditions, with and without $CaCO_3$, however a much higher intensity was observed in the case of the medium with $CaCO_3$.

5.2.3 - Rhodococcus erythropolis time-course

Afterwards the *Rhodococcus erythropolis* time-course was carried out for 96 hours, in shake flasks. Optical density readings were done at 24-hour, 72-hour and 96-hour incubation period. Results are presented in figure 9. It was concluded that from 24-hour incubation time onwards the bacteria didn't grow that much, leading us to understand that most likely the log phase would end shortly after the 24-hour growth, with approximately 1.6×10^9 cells/mL. It was also noted that in the shake flasks with the $CaCO_3$ (100 mg/mL), a decline of viable bacteria happened after the 72-hour incubation.

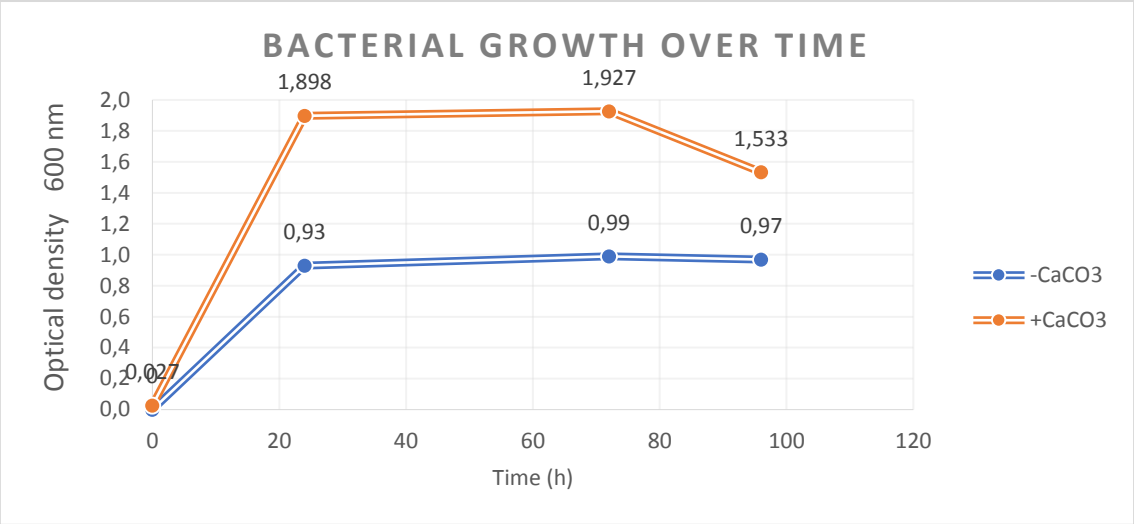

Figure 10 - Bacterial growth over time in the shake flasks, at 30 ºC, 150 rpm.

When comparing the growth data with the barely existing literature on this subject, it was possible to observe that at the 24 h mark the bacterial growth obtained in this work is similar, to the reported by Kurane et al. at the 24 hours mark it was observed an optical density around 1.0 ~ 1.2. [51].

The TL's production was evaluated by TLC, and a similar purple spot was observed in the media with $CaCO_3$, after 24 h and 72 h growth.

5.2.4 - Effect of inoculum concentration and culture stability

Shake flasks were used to establish the optimum inoculum growth conditions. In order to test the effect of inoculum concentration on *Rhodococcus erythropolis* growth and trehalose lipids production the following concentrations were tested: 1.0 % and 5.0 %. A similar growth profile was observed in the two inoculum concentrations tested. The cultures reached a stationary phase approximately after 24 h, with a maximum optical density of 2.7. (Table 7) and 2.2 x 10^9 cells/mL (culture 3), with an inoculum concentration of 5 %. However, an inoculum of 1 % allowed a similar cellular concentration, approximately 2.1 x 10^9 cellls/mL.

In these assays were also evaluated, the effect of culture storage at 4 ºC, on *Rhodococcus erythropolis* growth and TL´s production. This effect was evaluated after 6 and 3 months storage at 4 ºC, respectively.

Different shake flasks were done at different times, in order to determine which condition would be better – 1.0 % or 5.0 % of inoculum concentration, after storage.

The *Rhodococcus erythropolis* growth was carried out, after inoculation (5 %), for 24 h. After it was kept for 6 months at 4 ºC (culture 4). After that time a cellular concentration of 4.8 x 10^8 cells/mL was observed.

Similar assays were carried out with two different inoculum concentrations (1 % and 5 %), at 30 ºC for 24 h. After they were stored, at 4 ºC, for 3 months. Afterwards, the cellular concentrations obtained were respectively of 1.4 x 10^9 and 1.5 x 10^9 cells/mL, for 1 % and 5 %, inocula concentrations.

Table 6 - Rhodococcus erythropolis cells growth at OD600, of 5 different cultures in shake flasks, at 30 ºC, for 24 h.

Culture Number	Innoculum (% $_{v/v}$)	Optical density (600 nm)	*Rhodococcus erythropolis* concentration (cells/mL)	TL´s production (mg/mL)
1*	5	1.8	1.5 x 10^9	0.04
2	1	2.6	2.1 x 10^9	0.30
3	5	2.7	2.2 x 10^9	0.30
4*	1	1.7	1.4 x 10^9	0.05
5**	5	0.6	4.8 x 10^8	0.04

*These cultures were stored at 4 ºC for 3 months. ** The culture was stored at 4 ºC for 6 months.

Culture number 2 and 3 were analyzed in terms of glucose consumption, to establish which culture setting would be the best to produce trehalose lipids. Depending on the glucose consumption it was estimated that the trehalose lipid harvesting would be greater. DNS assay was then carried out as described in materials and methods (see 4.5.1).

With these readings and with the data from Figure 4 (DNS Calibration Curve), the graphic equation of the calibration curve was used to do the calculations. From the 10 mg/mL glucose concentration in the initial medium, only 0.70 mg/mL and 0.48 mg/mL remained, in (cultures 2 and 3, respectively). This led to the conclusion that more than 90 % of the

glucose was consumed, respectively 93 % and 95 %, which meant that the bacteria didn't need to grow more than 24 hours to proceed to the trehalose lipid extraction.

The TL's production was evaluated by TLC, for the 5 cultures (Table 7). It was observed similar spots for both innocula concentrations used, however, the 5 % concentrations, allowed more intense spots. Harshada et al. obtained similar results, as these authors also found that TL's produce a single purple-colored spot. [52]
The purple spot in TLC and a similar Rf (migration distance of substance)/(migration distance of solvent front) was observed for the TL´s obtained from the stored cultures (3 and 6 months). This was an important result, which means that the TL´s produced was stable at 4 ºC, for 6 months.

5.2.5 - TL's Extraction Process

The optimization of the extraction process from culture broth, includes different approaches. The first liquid-liquid extraction method attempted, was ethyl acetate:growth medium (1:1), as described in 4.5.6, but unfortunely it was not possible to isolate the TL's.

As described above, an extraction method of ethyl acetate:methanol (8:1) solution was used, and, after drying out the extracts with anhydrous sodium sulfate, samples were then collected. For the culture 2 a 0.3 mg/mL of TL´s was obtained (Table 7). A total of 120 mg (from 400 mL of culture broth) was collected to the 1st eppendorf in order to be prepared for the cytotoxicity test, a 2nd eppendorf was prepared for the HPLC, GC and NMR tests whilst a 3rd Eppendorf was collected for storage. A 4th Eppendorf with a slightly different colored final product that the HPLC ended up confirming that it was the same sample as the other ones, just with less intensity on its chromatography peaks.

5.2.6 - Separation and identification of TL's

Separation and identification of TL's were carried out under the TLC and HPLC conditions described, respectively in 4.5.3 and in 4.5.4.

TLC showed that the TL's gives a purple-colored spots with a similar Rf in the extracts of the *Rhodococcus* media as mentioned previously.

The HPLC method developed for the identification of TL's, presented in 4.5.4, allowed a preliminary identification of TL's. The samples of the TL's were solubilized in acetonitrile, in proportion of 1:4, however due the low solubility of the compounds they were not completely solubilized and when it was tried to increase the proportion of solvent, the reduction of TL's concentration was too low to be detected by the analytic method.

In Figure 10 is presented the peaks and the elution time for the TL´s from the samples of cultures 1, 2, 3 and 4 (Table 7). Retention times of 3.04 and 3.10 were observed for TL´s from cultures 2 and 3, while for culture 4 the time was 2.91 and 2.73. Similar retention times were obtained by Patil et al. [52]

In future work the TL´s solubility problem must be studied in order to have the compounds completely solubilized and the identification of the TL´s can be fully achieved.

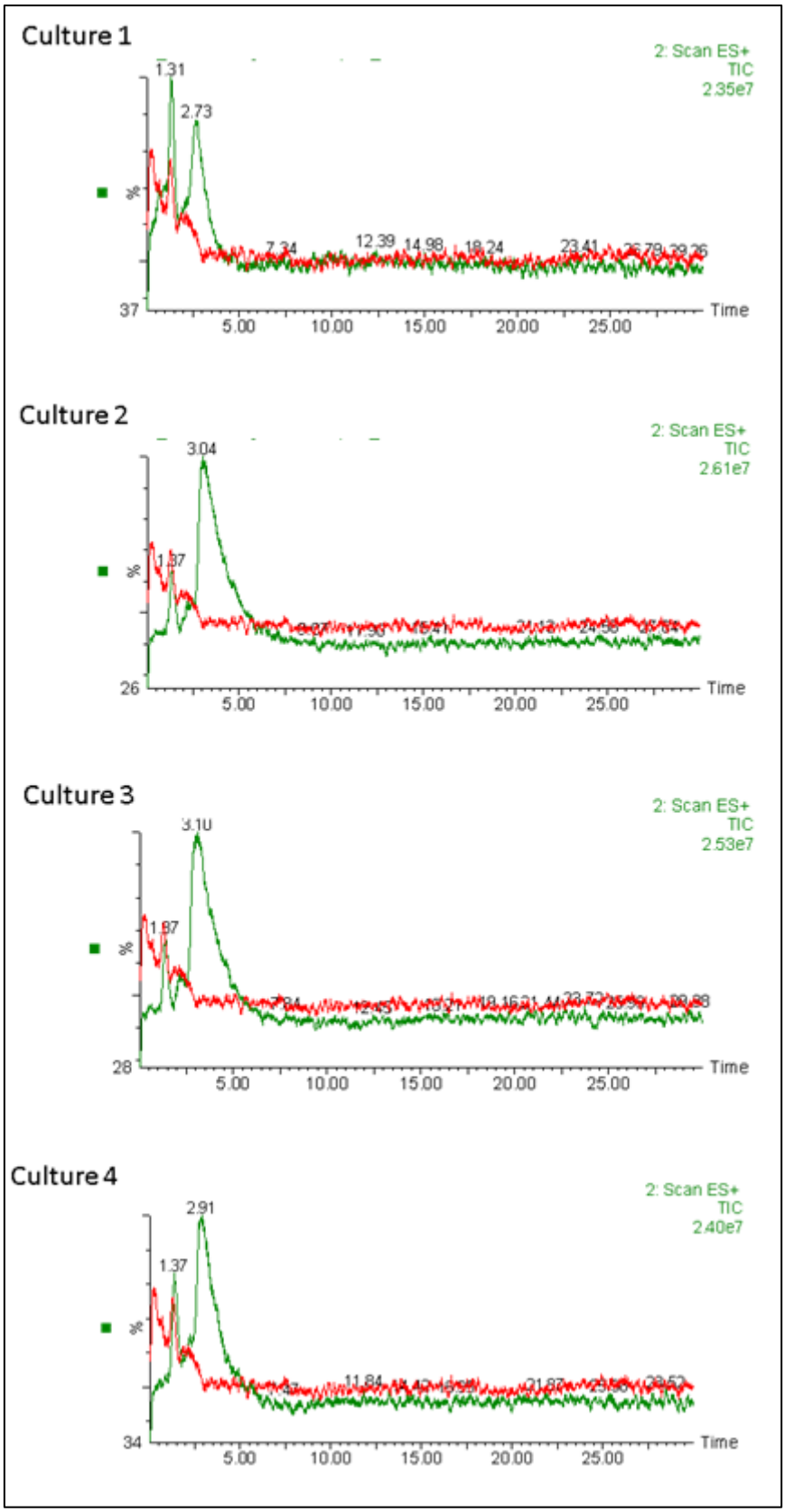

Figure 11 - Peaks and elution times for the TL's - HPLC (red – blank; green – extracts from the media).

5.2.7 - Cytotoxicity Screening

As it has been previously studied, some glycolipids can have anti-viral, anti-microbial and/or anti-tumoral activities. Trehalose lipids have been shown to have anti-viral properties against herpes simplex and influenza viruses. There is also literature showing that trehalose lipids have excellent growth inhibition and differentiation-inducing activities against human leukemia cells such as myelogenous leukemia cell K562, promyelocytic leukemia cell HL60 and basophilic leukocyte KU812. [53, 54, 55] Just like Matsumoto et al. [56] have done with trehalose liposomes against lymphoblastic leukemia. So, the last goal of this work, was to evaluate how cytotoxic can the trehalose lipid produced in this work be against cancer cell lines, and, as such, an initial screening was done with crystal violet assay.

Adherent cells detach from cell culture plates during cell death. This characteristic can be used for the indirect quantification of cell death and to determine differences in proliferation upon stimulation with death-inducing agents. One simple method to detect maintained adherence of cells is the staining of attached cells with crystal violet dye, which binds to proteins and DNA. Cells that undergo cell death lose their adherence and are subsequently lost from the population of cells, reducing the amount of crystal violet staining in a culture. [57]

In the first assay, there were problems with the solubilization of our TL product. In fact, the highest concentration possible to achieve without having precipitation, which cannot happen in this type of assay, was 12 mg/mL with distilled water at 25ºC and 5 minutes of ultra-sonication, with DMSO at 1 % v/v in the final concentration. Then, after undergoing the 1/10 dilution process, the starting concentration was 1,2 mg/mL. The total media used was 200 µL of DMEM so that the final concentrations of TL's were: 60 µg/µL, 40 µg/µL, 20 µg/µL, 10 µg/µL, 5 µg/µL and 1 µg/µL. Using media and DMSO 1 % (v/v) as negative controls and DMSO 10 %(v/v) as positive controls, after a 24-hour exposure time, it was not possible to get any results since there was no cell death.

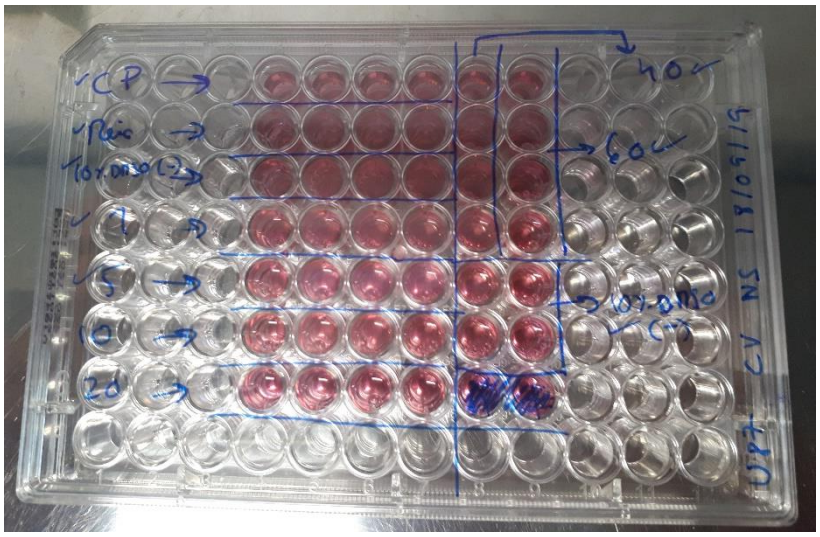

Figure 12 - First assay microplate with the concentrations drawn in the lid.

After this, it was decided to re-structure the cytotoxic assay. In order to do so, another solvent was used at the same time as DMSO, which was PBS. As PBS is not harmful to cells, it does not need to undergo the 1/10 dilution process and therefore can be directly added to the test system. The highest concentration reached with PBS as solvent was 88 µg/mL in the test system. So, given the fact that the concentration was not as high as it was expected, it was decided to extend the exposure time from 24 hours to a 48-hour period.

Two independent assays were done with the following concentrations (each one had quadruplicates as well the media and the positive control), with each solvent.

After the 48-hour exposure time, the crystal violet assay was performed, just like in the 1st trial, with the 24-hour exposure.

The absorbance was then read, in all the quadruplicates, in both microplates, and the results were the following, presented in figures 11 and 12.

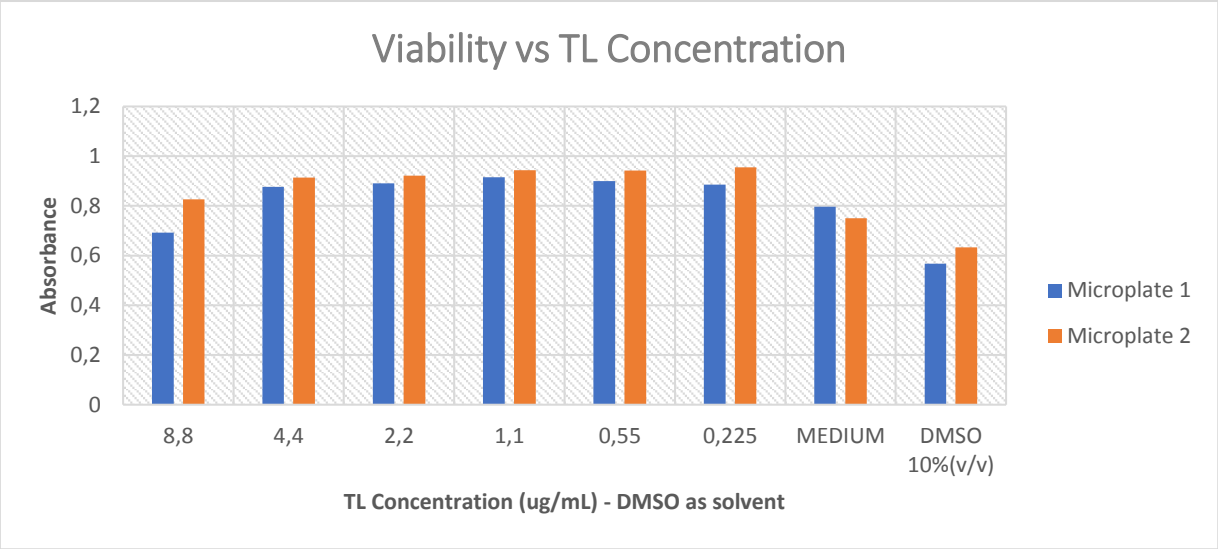

Figure 13 - Raw data with DMSO as solvent - Absorbance readings of DMSO as a solvent and Medium. Microplate 1 and microplate 2. (blue and orange, respectively). The assays are completely independent.

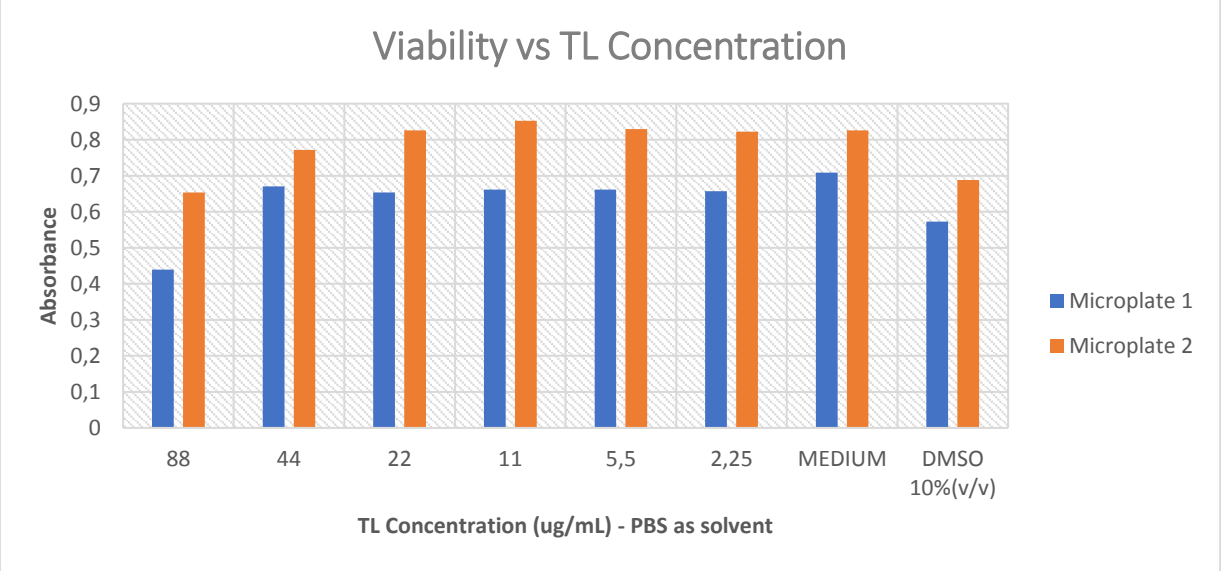

Figure 14 - Raw data with PBS as solvent - Absorbance readings of DMSO as a positive control and PBS as a solvent. Microplate 1 and microplate 2. (blue and orange, respectively). The assays are completely independent.

TL's in figure 11 and 12, showed that at the highest concentration with PBS as solvent was 88 µg/mL, as it showed major reduction of cell viability. After treating the data accordingly, the following dose response was observed, after excluding the outliers of our statistics, presented in figure 10. The same was for the 2nd microplate, showed in figures 11, 12 and 13.

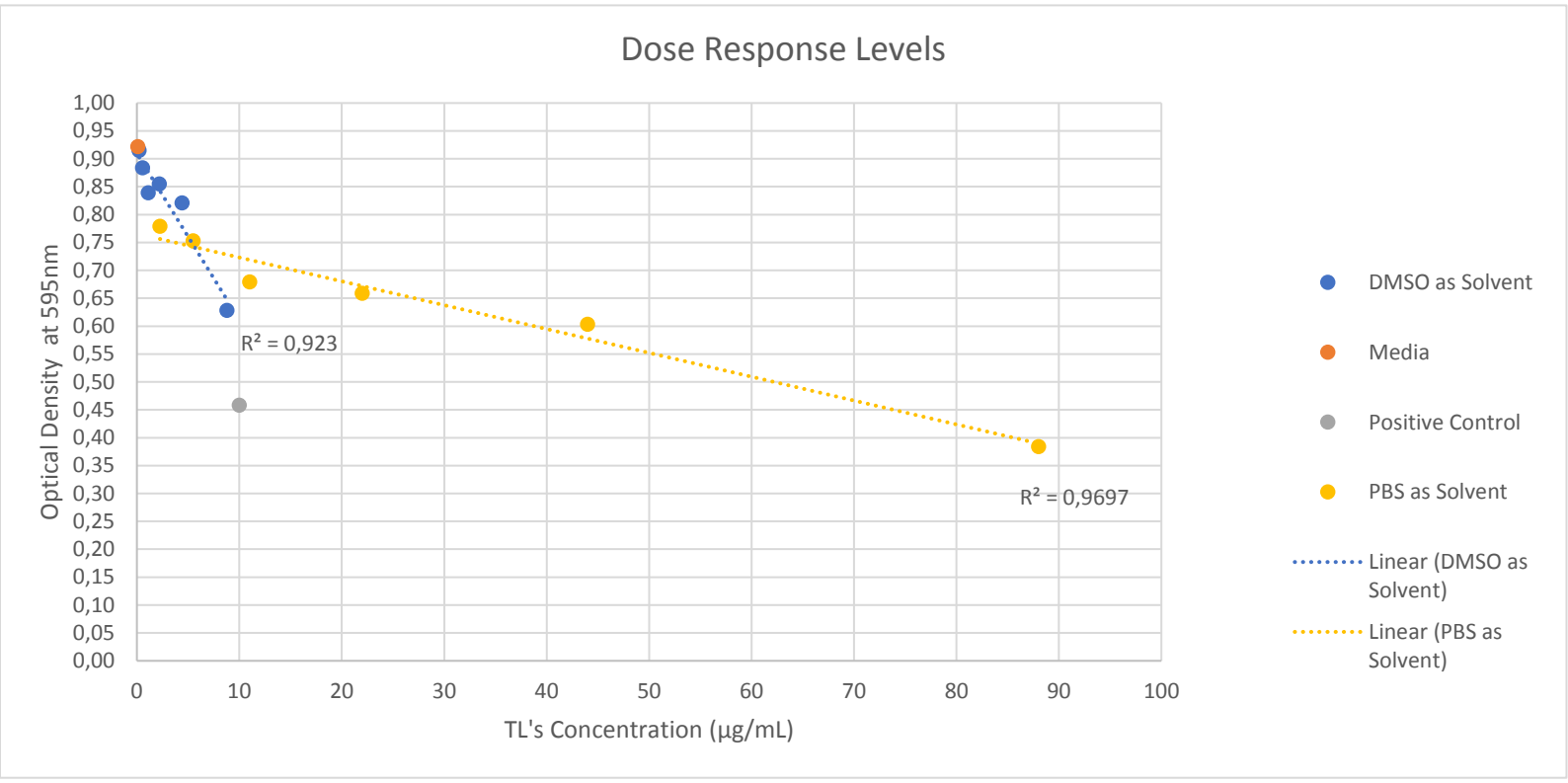

Figure 15 - Dose Response Levels from microplate 1, treated with statistically relevant data. (R2 higher than 0.9)

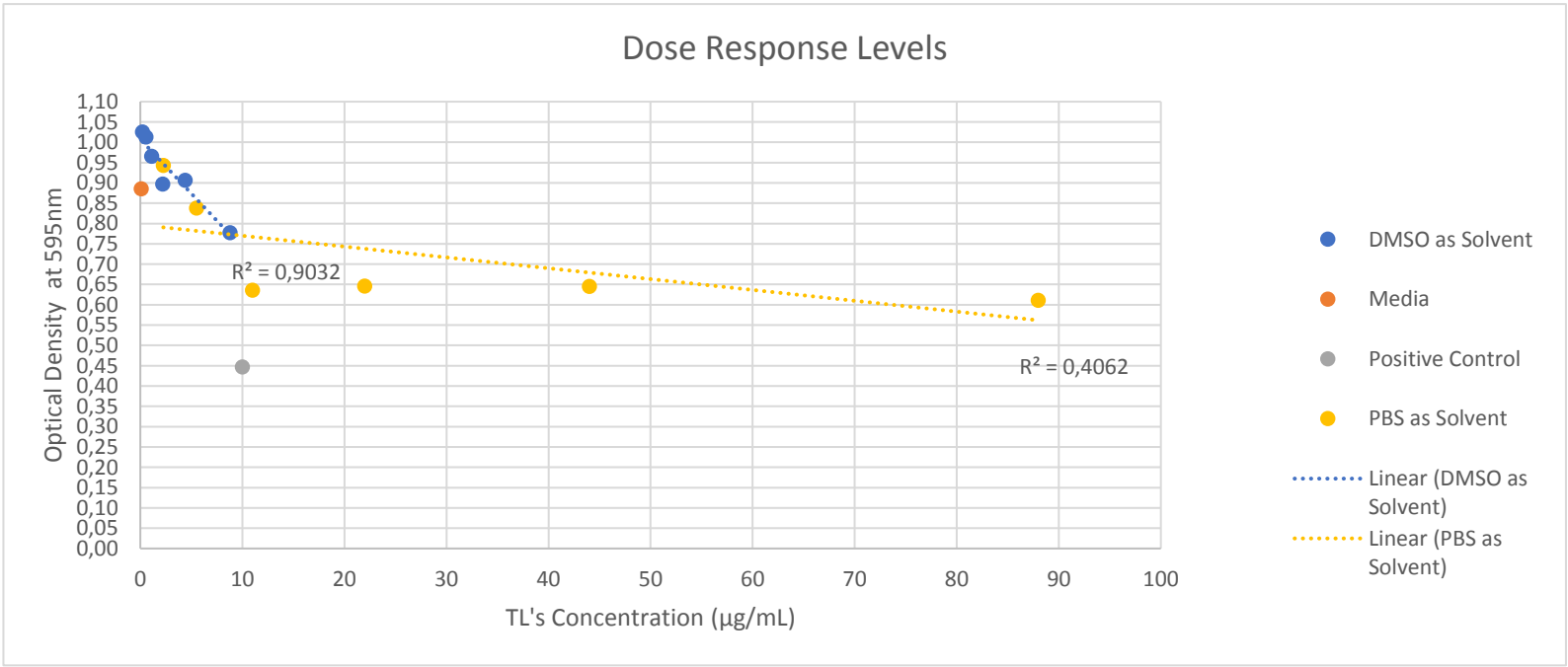

Figure 16 - Dose Response Levels from microplate 1, treated with statistically relevant data. (R2 higher than 0.9)*

* With the PBS solutions it was not possible as from 11 µg/mL onwards the cytotoxicity effects seen were pretty much the same.

Even though that in the 2nd assay it was not possible to have a proper Dose Response curve, it is possible to see cytotoxicity effects. When comparing the values directly with the cell viability seen in the non-treated cells, it is possible to address the following conclusions, shown in table 10. In both microplates the cell viability was reduced by at least 30 % when compared with non-treated cells and, in microplate 1, it was possible to achieve a cytotoxicity level higher than the positive control, which clearly indicates that the TLs are having a cytotoxicity effect against the cancer cell line tested (U-87).

Table 7 - Viability results after directly comparing with the non-treated cells.

	Viability (%)	
Controls / TL's	Microplate 1	Microplate 2
Medium	100	100
88 ug/mL (PBS as a solvent)	41.6	69.0
8,8 ug/mL (DMSO as a solvent)	68.1	87.8
DMSO 10 % (v/v)	49.7	50.5

6 - Conclusion

These studies demonstrate that it is possible to produce enough trehalose lipid product in a 24-hour growth period, using a 24-well microplate or a through shake flasks. Using a not expensive medium, it was possible to produce 120 mg of trehalose lipids, using 1 % (v/v) of bacteria in YM Medium supplemented with glucose, in roughly 24h. Furthermore, the extraction process used takes no more than a day and the compound was ready to be tested in any test system.

Solubilization of the compound was very difficult to accomplish, as these types of glycolipids show a reduced solubility in most aqueous systems and in some organic solvents. Therefore, it was not possible to dissolve TL's in water, in DMSO the highest concentration was 8.8 µg/mL and with PBS was 88 µg/mL. It is known that it is easily soluble in solvents such as methanol, but it was not possible to use those solvents in these types of tests as the solvent needs to be as harmless as possible to our test system.

With crystal violet initial screening, it was proved that the produced trehalose lipid had a cytotoxic effect in the specific U-87 cell line which accounts for a human glioblastoma, opening a perspective on how these natural products can be used against oncological diseases. Further studies should rely on trying to obtain more concentrated solutions of this glycolipid, reduce the solubility problems, as well as testing it in different cell lines.

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