Rui Pedro Cordes Bento Coelho de Matos

Licenciado em Bioquímica

Fermentation of white grape pomace by *S. bombicola* for the production of sophorolipids

Dissertação para obtenção do Grau de Mestre em Biotecnologia

Orientador: Prof. Madalena Salema Oom, IUEM-UCIBIO
Coorientador: Prof. Susana Filipe Barreiros, FCT-UNL

Júri:

Presidente: Prof. Doutor Rui Oliveira

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Resumo

A necessidade de reutilização de resíduos agroindustriais é uma prioridade. O objetivo deste trabalho foi reutilizar o bagaço da uva branca (WGP), um resíduo lignocelulósico rico em hidratos de carbono (cerca de 42 % m/m de açúcares solúveis livres) e óleo (cerca de 13 % m/m) como fonte de carbono para a produção de soforolípidos (SL) por uma levedura, *Starmerella bombicola*, conhecida por sobreviver em meios com elevadas concentrações de açúcar e por ser uma levedura frutofílica, usufruindo igualmente da presença de ácidos gordos no meio de crescimento. Os soforolípidos são biosurfactantes conhecidos por serem biodegradáveis, biocompatíveis e com grande valor acrescentado nas suas características como surfactante, que também demonstram atividade anti tumoral e antimicrobiana. Os açúcares livres de bagaço de uva branca foram obtidos por extração com água enquanto os óleos foram obtidos por extração de CO_2 supercrítico ($scCO_2$) assim como por extração de Soxhlet.

As experiências de crescimento de leveduras levaram a uma concentração máxima em soforolípidos de 34,9 g/L, num meio que continha hidratos de carbono livres e óleos de bagaço de uva branca. Os soforolípidos foram purificados com n-hexano e caracterizados através de LC-MS, ^{1}H-RMN e medições de tensão superficial. Ambos os tipos de soforolípidos, lactónicos e acídicos, tanto com ácido linoleico como com ácido oleico na sua estrutura, foram detetados na análise de LC-MS. Estas estruturas eram também as mais abundantes no óleo de bagaço de uva branca: ácido linoleico (62 %) e ácido oleico (17%). A análise de ^{1}H-RMN também detetou a presença de caudas de hidrocarbonetos C18 e soforose. Os soforolípidos obtidos tinham uma tensão superficial mínima (MST) de 35,62 mN/m para uma concentração micelar crítica (CMC) de 53,79 mg/L.

Palavras-chave: bagaço de uva branca, dióxido de carbono supercrítico, soforolípidos, *Starmerella bombicola*, extrato de açúcares livres, extrato de óleos.

Abstract

The use/valorization agro-industrial waste is a priority worldwide. The objective of this work was a novel valorization of white grape pomace (WGP), a lignocellulosic waste feed-stock rich in carbohydrates (around 42 wt. % of free soluble sugars) and oil (around 13 wt. %). The objective was to use the WGP free sugars and oil as carbon sources for the production of sophorolipids (SL) by *Starmerella bombicola*, a yeast species known for thriving in sugar-rich environments and being fructophilic, while equally comfortable with the presence of oils in growth medium. The goal was to produce sophorolipids (SL), bio-surfactants known to be biodegradable, biocompatible and with high added value surfactant characteristics, which also exhibit anti-tumor and antimicrobial activity. Free sugars of WGP were obtained by water extraction and oils of WGP was obtained by supercritical CO_2 extraction and also by Soxhlet extraction.

Yeast growth experiments led to a maximum sophorolipid yield of 34.9 g/L with a medium containing both WGP free carbohydrates and oil. Sophorolipids were purified by n-hexane extraction and characterized using LC-MS, ^{1}H-NMR and surface tension. Both types of sophorolipids, lactonic and acidic, with both linoleic acid and oleic acid moieties, were detected in LC-MS analysis. These moieties were the most abundant in WGP oil: linoleic acid (62 %) and oleic acid (17 %). ^{1}H-NMR also detected the presence of C18 hydrocarbon chain lengths and sophorose. The SL obtained had a minimum surface tension (MST) of 35.62 mN/m for a critical micellar concentration (CMC) of 53.79 mg/L.

Keywords: white grape pomace, supercritical carbon dioxide, sophorolipids, *Starmerella bombicola*, free sugar extract, oil extract.

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^{1}H-NMR – Proton nuclear magnetic resonance

acSL – Acidic sophorolipid(s)

$CDCl_3$ – Deuterated chloroform

CO_2 – Carbon dioxide

dH_2O – distilled water

FAO – Food and Agriculture Organization

FT-IR – Fourier transform infrared spectroscopy

GC – Gas chromatography

GP – Grape pomace

HPLC – high-performance liquid chromatography

lacSL – Lactonic sophorolipid(s)

LC – Liquid chromatography

MALDI-TOF MS – Matrix assisted laser desorption/ionization time-of-flight mass spectrometry

MS – Mass spectrometry

m/z – Mass/charge ratio

OCS – Oil carbon sources

RGP – Red grape pomace

$scCO_2$ – Supercritical carbon dioxide

SCS – Sugar carbon sources

Sg/YE ratio – Sugar concentration/yeast extract concentration ratio

SL – Sophorolipid(s)

TLC – Thin-layer chromatography

YE – Yeast extract

WGP – White grape pomace

1 State of the Art

1.1 Agriculture and agroindustrial wastes

Agriculture and agroindustry residues are one of the top environmental concerns of society (Ferrari et al., 2018). The usual alternatives of reutilization proposed for these residues are biodiesel production, biomass-generated energy, fertilizer and feeds for all types of cattle due to their highly nutritive contents and energy potential. Nonetheless, agriculture and agroindustry continue to be one of the leading causes of pollution being responsible for around 38% of all water pollution in the EU as well as being, in the United States, the leading source of pollution in rivers and streams, second main source in wetlands and third source in lakes (Food and Agriculture Organization, 2017). Because of this, the possibility to attain further value-added products from these continues to arise the curiosity of the scientific community as more and more articles and studies preponderate, discussing the need for self-sustaining and green methods of repurposing residues that are usually discarded and not cared for by industries and can cause several types of pollution and serious environmental issues. Common sense states that these wastes should be reutilized as they may offer a very interesting and sustainable ecosolution for many environmental problems that our planet faces (Beltrán-Ramírez et al., 2019).

The wine making industry is one of such industries. Every year, it produces tons of by-products that are either deposed of in landfills, sent to composting, incinerated or simply discarded unattentively (Muhlack et al., 2018). In 2018 alone, the European Union is estimated to have reached 181.9 million of hectoliters, a rise of 28.3 % when compared to 2017 (OIV, 2019). A world map with statistical data about grape production is represented in **Figure 1.1**. This roughly translates into more than 20% of solid waste by-products (Yu et al., 2013) which is equivalent to, more or less, 18.2 million tons of waste-by-products. One of these solid residues of wine industry is grape pomace (GP) - it is comprised of seeds, skins, stalks and pulp that are leftovers from the wine making process and are considered a major pollutant of waterways and soils in countries with a large tonnage of wine production. Untreated grape pomace can be the cause of pests, organic and even chemical wastewater discharges as well as oxygen depletion in soils and underground water infiltrations (Gómez-Brandón et al., 2019). These residues became an issue when the market of grape spirit, usually made from GP, plummeted, while wine production continues on the rise. Discarding GP became an economical loss for industries leading to a search for effective ways of reducing this net loss by using it as substrate to produce value-added products (Botelho et al., 2018).

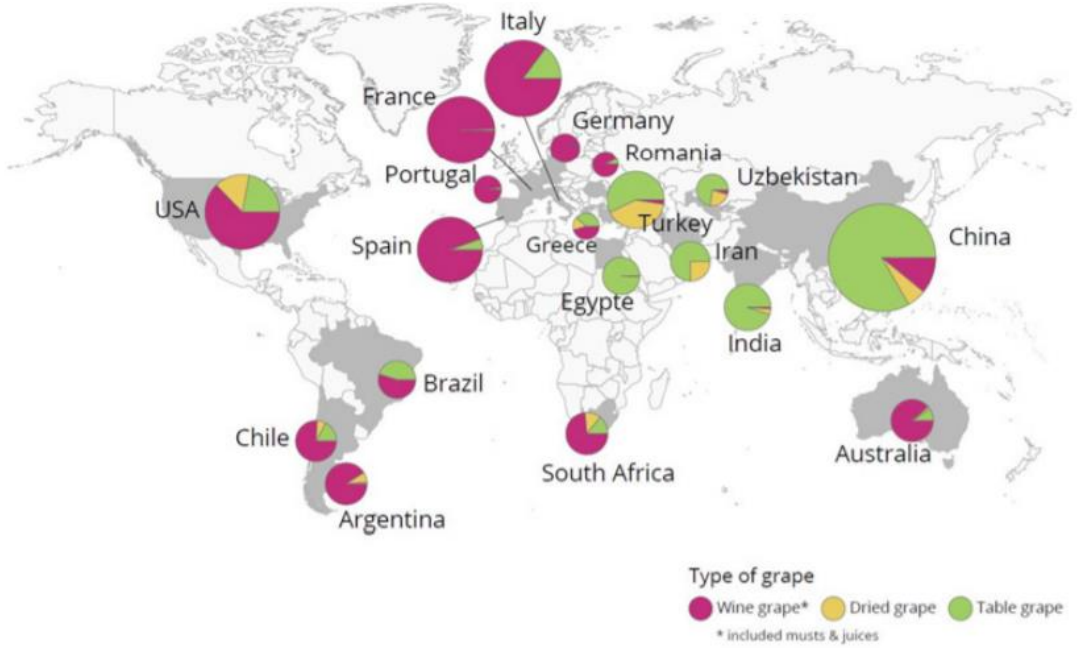

Figure 1.1 - Statistical map of grape production around the world in 2019. Retrieved from OIV 2019 Statistical Report on World Vitiviniculture (OIV, 2019 at http://www.oiv.int/public/medias/6779/state-of-the-vitiniculture-world-market-oiv-2019-congress-pr.pdf)

GP contains a large amount of natural bioactive compounds that can be reutilized or valorized in various industries or primary activities, from pharmaceuticals to cattle feed and food supplements. Phytochemicals such as phenolic acids, flavanols, flavonols, anthocyanins and others are highly present in GP and have been shown to possess intrinsic beneficial effects for human health such as prevention of cardiovascular diseases, anti-inflammatory behavior or anti-oxidant activity (Negro et al., 2003; Saura-Calixto, 1998; Shrikhande, 1999). Because of this nutritive and therapeutic richness, the scientific community has been exploring, throughout the last two decades, how these compounds can be effectively extracted and what further uses can be given to GP. For instance, studies performed on extracted compounds from red grape pomace (RGP) showed that a large amount of polyphenols are present within it and are enough to exert anti-hypertensive effects on spontaneously hypertensive rats (Rasines-Perea et al., 2018).

Also, an enzymatic inhibition study on α-glucosidase of rats with induced diabetes revealed that both RGP and white grape pomace (WGP) have significant quantities of antioxidants and anti-hyperglycemic compounds to be considered complementary therapies or at least helpful in maintaining glucose levels in Type 2-diabetes patients (Hogan et al., 2010).

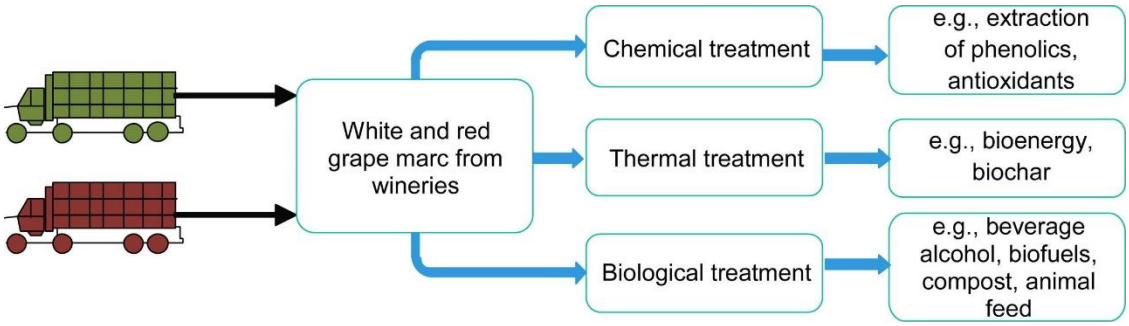

Figure 1.2 - Treatment procedures for grape pomace/marc residues of wineries (Muhlack et al., 2018).

Grape marc (**Figure 1.2**) is composed of only the skins and pulp of grapes and is usually considered to be part of grape pomace as a whole, their fates in the treatment procedures being the same. However, and despite all these perspectives for the reutilization of GP, there are a few certain aspects that remain relatively unexplored.

1.2 Novel valorization of white grape pomace

As mentioned before, GP is currently the object of study for various applications. In order to respect the tenets of sustainable chemistry (**Figure 1.3**), typical extraction methods used to obtain natural products are non-organic and alternative solvents such as subcritical water, supercritical carbon dioxide and eutectic solvents or even non-solvent approaches like microwave hydrodiffusion and gravity and pulse electric field (Chemat et al., 2019). RGP is well known for having a large composition in polyphenolics as opposed to WGP, which is certain to contain higher amounts of soluble sugars (Dwyer et al., 2014). This fact occurs due to the different production methods of red wine and white wine, e.g. RGP is retrieved after the fermentation procedure whereas WGP is set aside before the fermentation itself (**Figure 1.4**). As such, WGP has higher concentrations of soluble

Figure 1.3 - The twelve Green Chemistry principles (Frolich Institute, https://frolichinstitute.org/green-chemistry-1).

sugars because it has not suffered any type of fermentation while RGW has a much lower free sugar content but a higher concentration of one other group of interesting biomolecules, polyphenolics, and products of fermentation (Infovini, 2019). Out of these alternative and green methods, supercritical carbon dioxide ($scCO_2$) is perhaps one of the most effective in retrieving oils and polyphenols out of GP.

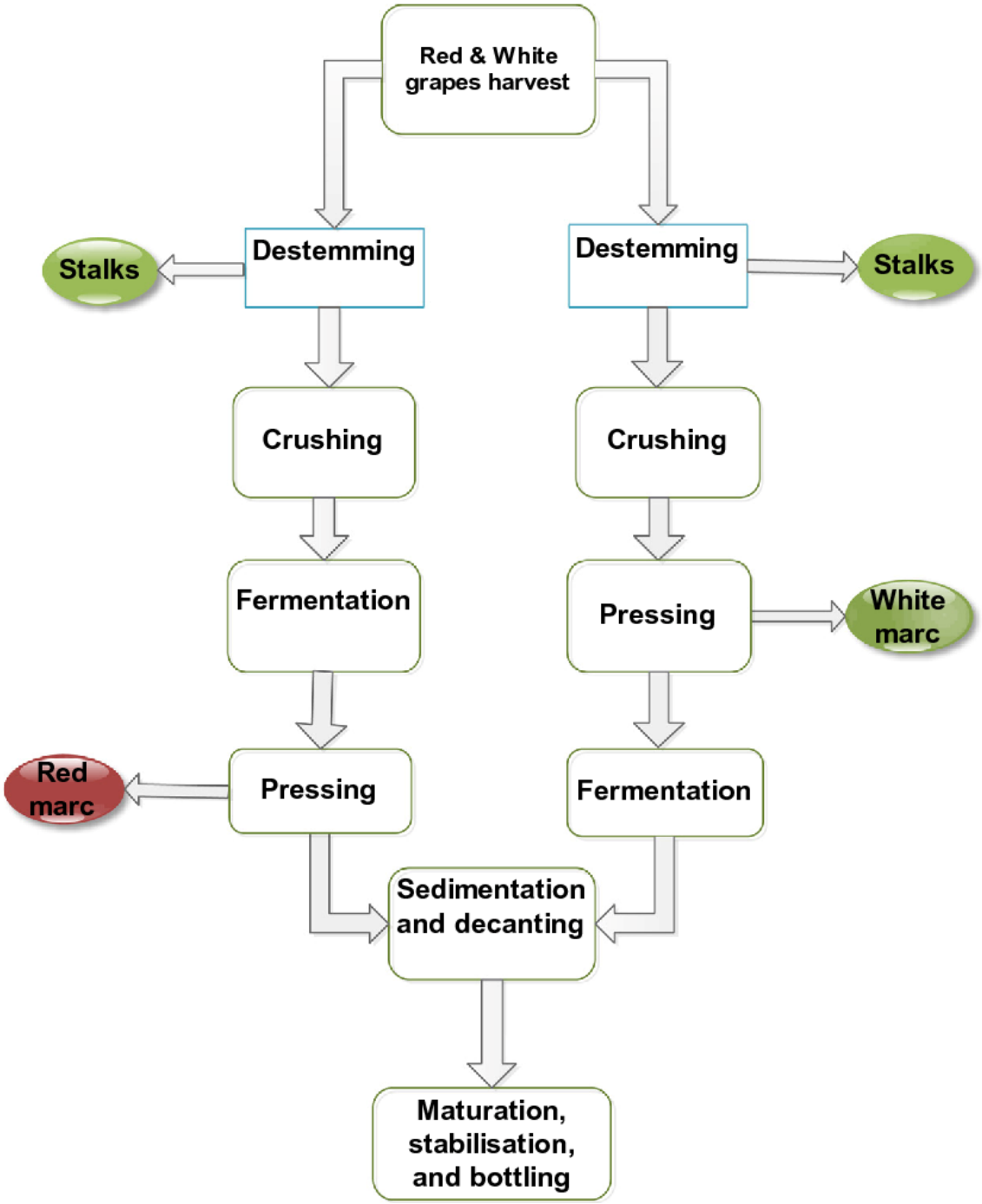

Figure 1.4 - White and red grape processing in the wine making industries. The stalks and the marc together make up the composition of grape pomace. Adapted from Muhlack et al., 2018.

Numerous authors have pointed out the most effective and optimized procedures for $scCO_2$ with the goal of extracting as many of these nutrients and chemicals as possible. As an example, a team in Trento, Italy, performed solubility experiments with $scCO_2$ in an attempt to optimize the conditions of extraction of essential lipids from grape seeds and compared their thermodynamic model to density-based models that were described in-depth, in other literature (Duba and Fiori, 2016). Through extractions at various temperatures and pressures, they were able to determine the best conditions for extraction, achieving yields that were quite promising for future revalorization of GP by obtaining practically all of the content in oil of its seeds. Another team, also in Italy, worked specifically with white grape seeds in a two-step co-solvent supercritical fluid extraction process and obtained promising results in extracting proanthocyanidins and polyphenolics efficiently (Porto and Natolino, 2017).

Despite all the successes and continuous exploitation of WGP, it still withholds value in other components. Its high free sugar content, albeit well known, appears to be mostly directed at the production of biofuels or ethanol (Kalli et al., 2018).

1.3 Biosurfactants and their characteristics

Biosurfactants are molecules that are biologically produced by several strains of yeast, bacteria and fungi. They have active surface properties and have both hydrophilic and hydrophobic moieties and thus are considered to be amphiphilic molecules, with micellization capabilities. Apart from that, they are known to have great specificity, low toxicity and can be produced fairly easily, in accessible conditions, since they are extracellular entities. These biomolecules are being used in several industries such as mining, metallurgy, cosmetics and pharmaceuticals, and many more (Akbari et al., 2018). Their applicability in several industries speaks for the capabilities of these molecules; biosurfactants can be used as emulsifiers and demulsifiers, ingredients in food, detergents, foaming, wetting and spreading agents. Beyond all those characteristics, they have also been shown to be capable of bioremediation in environments that suffered large crude oil spills, even allowing for the oil to be recovered to a large extent in experiments using *Acinetobacter venetianus* ATCC 31012, that produced an emulsion that at just 0.5 mg/mL was capable of removing up to 98 % of crude oil reabsorbed to samples of limestone (Peele et al., 2018).

The sheer environmental value of these biomolecules is extremely high, so much so that biosurfactants are once again being extensively studied to replace common use detergents, as current commercial detergents are becoming more and more of an environmental threat due to their bulk presence all across the globe and the problems presented when eliminating their traces from water sources (Giagnorio et al., 2017; Rebello et al., 2014).

Biosurfactants can be effectively divided in four main chemical types, the most commonly known being glycolipids followed by phospholipids, lipopeptides and polymeric forms. Three of these types are displayed in **Figure 1.5**.

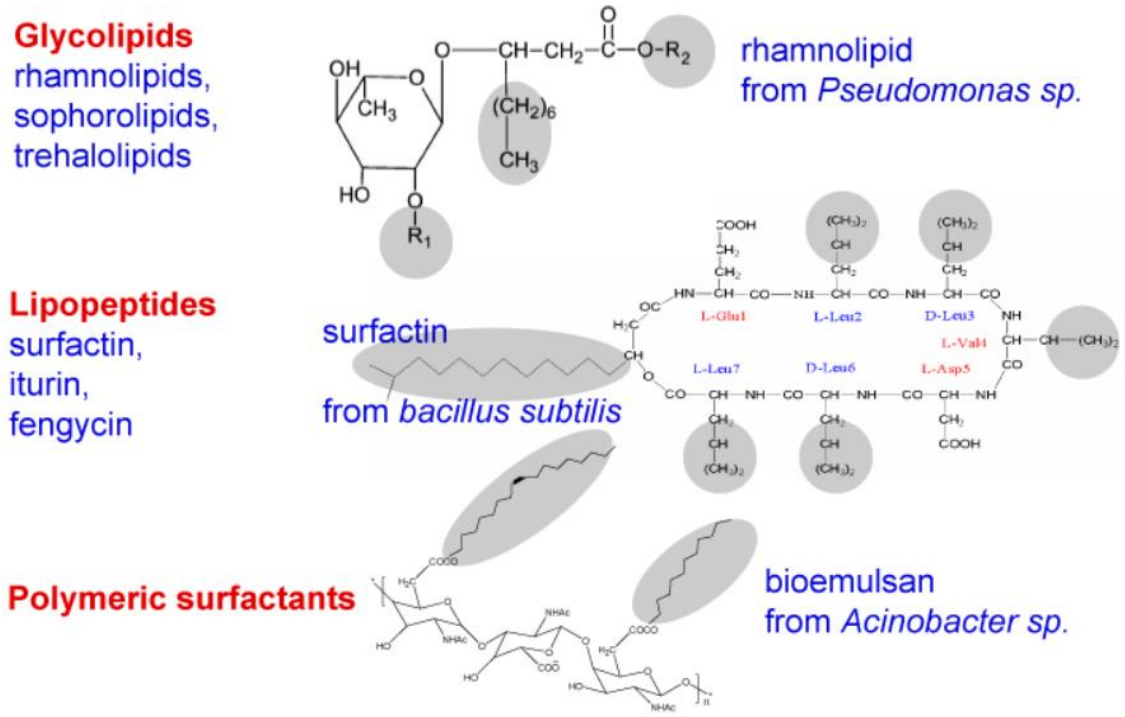

Figure 1.5 - Three of the four classes of biosurfactants – glycolipids, lipopeptides and polymeric surfactants - with illustrated examples of each group. Adapted from The Thomas/Penfold Group – Biosurfactants web page (http://rkt.chem.ox.ac.uk/projects/biosurfactants.html).

Rhamnolipids are one of the most studied biosurfactant molecules. They are known to have very interesting characteristics such as a lower critical micelle concentration and better bioavailability than common synthetic surfactants (Oluwaseun et al., 2017). Rhamnolipids have a tendency to have small hydrocarbon chains and to form dimeric molecules, that could have a higher than average bioactivity when one's goal is, for instance, anti-bacterial biofilm effect (Aleksic et al., 2017). Nevertheless, the highest production yields of rhamnolipids are usually obtained with pathogenic bacteria such as *Pseudomonas aeruginosa* (Li et al., 2019*;* Naughton et al., 2019) which need to be genetically manipulated in order to be certified for use in more commercial applications.

Trehalolipids, or trehalose lipids, are glycolipids produced by extremophilic microorganisms such as *Rhodococcus fascians* that have been under study for their particular interesting action in preventing adhesion of microorganisms like *Enterococcus faecalis*, *Escherichia coli* and *Candida albicans* to common materials like polystyrene and silicone while possessing antimicrobial action, prompting them to be used as antimicrobial material coatings (Aleksic et al., 2017). When looking at this perspective, it is clear that their use can become essential to keep sterility in medical equipment and materials and prevent hospital infections which are seen as one of the largest threats to healthcare services around the world.

Sophorolipids are extensively known for their potential to solve problems that are very serious, such as the ever increasing cancer incidence levels and excessive use of surfactants of synthetic origin, in a society that relies more and more on green and sustainable production methods (Callaghan et al., 2016; Mohamed, 2017).

1.4 Sophorolipids: chemical properties and variants

Sophorolipids (SL) are biosurfactant molecules with a particular general structure; they are typically composed of a glucose di-saccharide, sophorose, the glucoses being connected via an unusual β-1,2 bond, with a possible acetylation on carbon 6', 6'' or both at the same time. The lipidic portion of SL is usually a 16 or 18 long carbon terminal or subterminal hydroxy fatty acid bonded to either one of the glucoses of the sophorose molecule via a β-glycosidic link. Alternatively, at the carboxylic end of the long 16 or 18 hydrocarbon chain of the fatty acid, SL molecules can have one of two situations occurring; one being that it is free of any bonds conferring the SL an acidic nature (commonly called acidic SL) and the other being the formation of a cyclic, lactonic structure where the carboxylic end forms an ester bond with either the 4'' carbon or, occasionally, with the 6' or 6'' positions (commonly called lactonic SL). These modifications are all explicitly displayed in **Figure 1.6**. Adding to this, sophorolipids have better biosurfactant characteristics than most other molecules of the same type. They are known to have high stability in a wide range of pH, temperatures and salinity conditions while possessing excellent detergent properties, being low-foaming agents with wide interfacial properties. Moreover, under the right conditions, they are produced in large quantities and, like previously mentioned, even when using media with components usually rendered as residues of agroindustry (Oliveira et al., 2015).

Sophorolipids have been produced on all sorts of hydrophilic and hydrophobic carbon sources from the likes of sugar cane molasses, sweet sorghum bagasse together with common alkanes, soybean oil, sunflower oil, industrial fatty acid residues, and even waste motor oil (Huaimin et al., 2018). The variety of carbon sources that *S. bombicola* is capable of using to produce such a value-

added product makes it possibly one of the best biological chassis there is. As long as there are fatty acids and sugars to consume, *S. bombicola* is likely to produce SL to a certain degree.

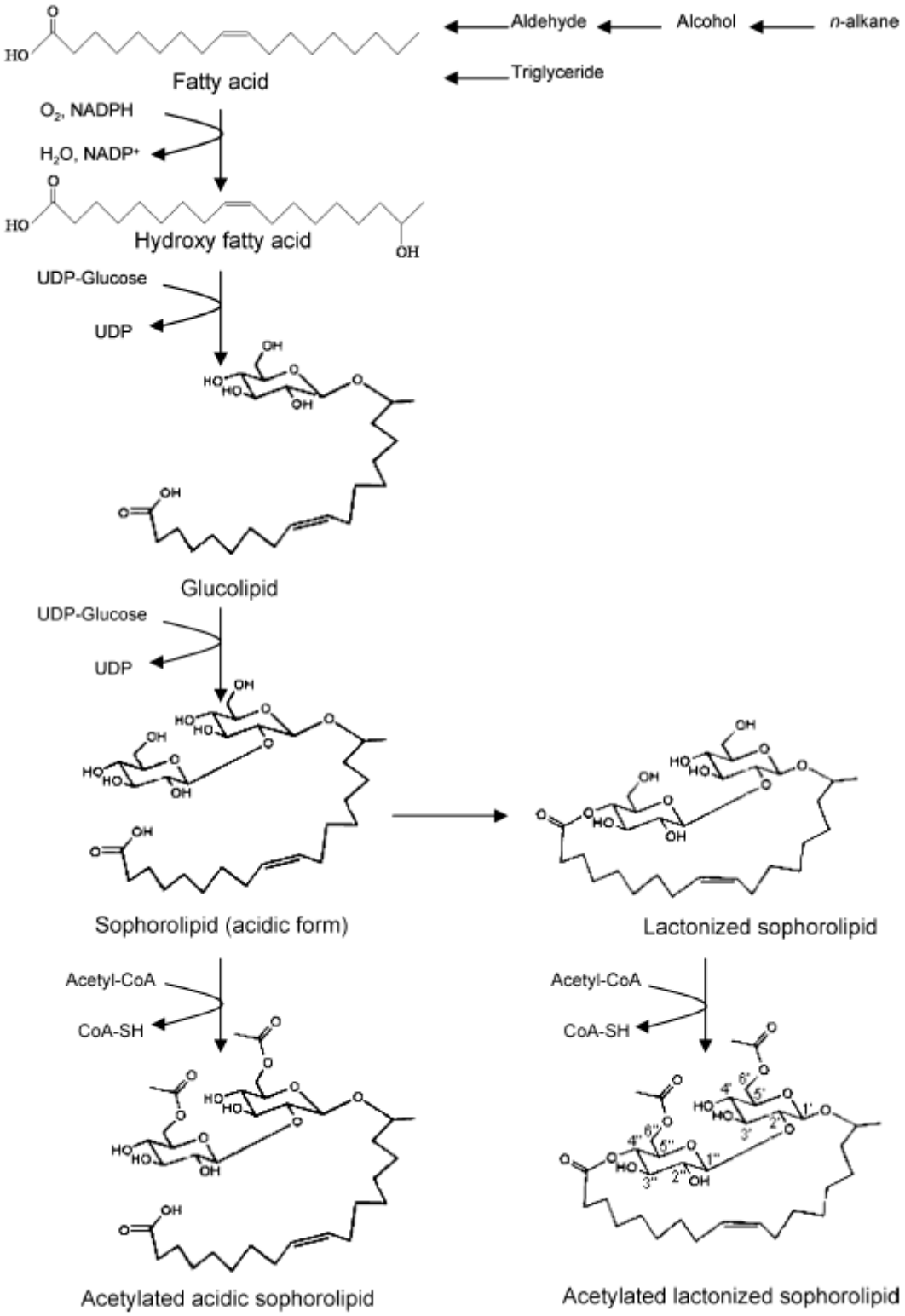

Figure 1.6 – General representation of acidic and lactonic SL, both acetylated and un-acetylated, and their usual anabolic pathway. Adapted from Saerens et al., 2007.

For instance in 1994 with *S. bombicola* on a medium composed of rapeseed esters and glucose, Davila et al. claim to have achieved a yield of 340 g/L after 190 h of fermentation (Davila et al., 1994). All of this shows to prove that sophorolipids are the gold-standard on easy to produce and highly valuable biological products with tremendous potential on their own.

1.5 Production of sophorolipids by *Starmerella bombicola*

Starmerella bombicola is by far the most studied yeast when SL production is the main goal of the authors. *S. bombicola* is non-pathogenic (an essential criteria for its use in widespread commercial products), the growth conditions are easy to reproduce and the yields in product are very high, even when using low-cost fermentation media with only sugar cane molasses, yeast extract, soybean oil and urea (Daverey and Pakshirajan, 2009). With such a media, Daverey and Pakshirajan (2009) were able to reach a production of 63.7 g/L in batch fermentation with 100 g/L soybean oil, 100 g/L sugar cane molasses, 10 g/L yeast extract and 1 g/L urea after only 60 hours while other authors managed to go up to 320 g/L of production after extending the production to 190 h, albeit in a 4 L fermentation reactor with constant feeding of substrates and using a much more complex production medium (Davila et al., 1992)

Several attempts of fully unveiling the metabolic properties of *S. bombicola*'s SL synthesis pathways have been done. Many authors have tried to bioengineer this particular species of yeast in order to increase SL yields and even modify their usual structural aspects. For instance, a group of authors were able to determine the level of specificity for *S. bombicola*'s glucosyltransferases, UgtA1 and UgtB1, the first and second glucosyltransferases interfering in the SL synthesis process when binding both glucoses that comprise the sophorose using deletion mutants (Saerens et al., 2015), providing further insight on how to manipulate this yeast species' genome and how the biosynthesis pathway functions (**Figure 1.7**).

Typically, extracellular SL are lactonic in their chemical nature, however some authors have stated finding acidic SL's when analyzing mass spectrometry results of SL retrieved after solid fermentation (Jiménez-Peñalver et al., 2018) as well as in some liquid fermentation media (Hu and Ju, 2001).

This yeast is also known to produce SL from fatty acid backbones synthetized "de novo". *S. bombicola* is considered an oleaginous yeast despite the lack of quantitative information when in regard to intracellular accumulation of lipids. The presence of an ATP citrate lyase has been shown as far back as 1996 and ultimately confirmed by genomic assays of acl1 and acl2 genes (De Graeve et al., 2018). This means that *S. bombicola* supposedly tends to store more than 20 % (w/w) triglycerides which can indicate that not all of the fatty acids synthetized or integrated by the yeast are directed towards the production of SL.

Nevertheless, as interest in SL and this yeast species in particular is on the rise, new data about *S. bombicola*'s metabolome, genome and proteome will surely be obtained in the near future.

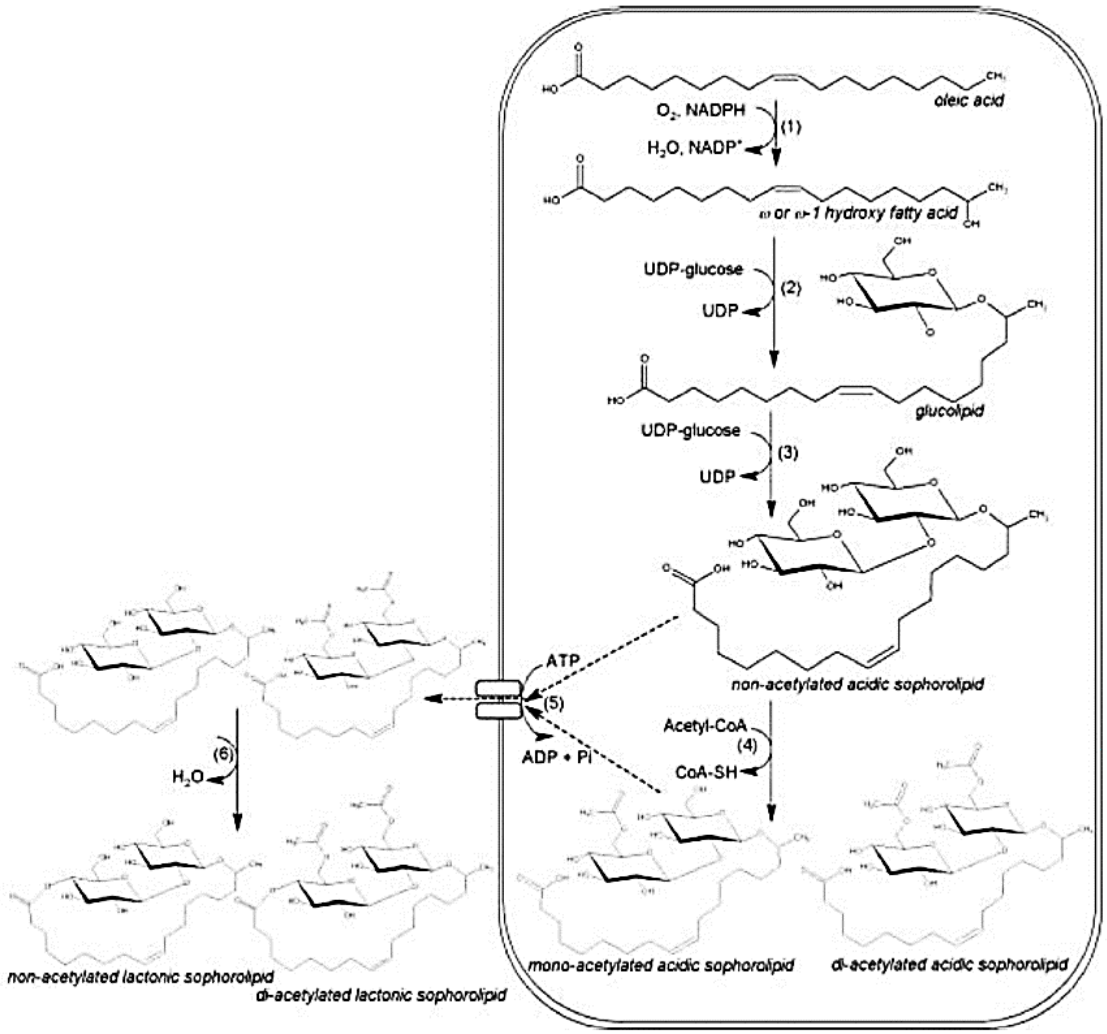

Figure 1.7 - Detailed schematics of SL biosynthesis pathway of *S. bombicola*. (1) Cytochrome P450 monooxygenase CYP51M1, (2) UDP-glucosyltransferase UGTA1, (3) UDP-glucosyltransferase UGTB1, (4) Acetyltransferase A1, (5) Sophorolipid transporter MDR, (6) Lactone esterase SBLE. Retrieved from Saerens et al., 2015.

1.6 Sophorolipid characterization methods

SL possess various structural and chemical forms, like previously explained. On account of this, characterization methods for this particular glycolipid are several. Additionally, production of SL tends to result in a complex mixture of molecular structures due to the variety in fatty acid moieties that producing microorganisms can incorporate in them. Because of this, methods that combine both a quantitative and a qualitative dimension tend to be the most effective in successfully characterizing these molecules, several authors regularly using a combination of more than one of these methods.

When obtained from fermentation media with no hydrophobic carbon sources, SL tend to be considered pure because it is unlikely that other molecules that are hydrophobic are present in an aqueous media, when recovered with organic solvents such as ethyl acetate. However, if SL originate from a fermentation media with supplements (fatty acids, hydrocarbons, minerals, etc.), additional

purification steps are required. Washing the produced SL with hexane a number of times, removing oils and remnant fatty acids, seems to be enough for some authors to consider them pure (Geys et al., 2018). Additionally, if minerals are present or if one wants to separate acidic from lactonic SL, other methods might be required such as crystallization and the use of aqueous solvents in separation (Hu and Ju, 2001). After the purification steps, typical approaches by authors are to characterize their product to some degree.

Figure 1.8 - Two of the analytical machines typically used in characterization of sophorolipids. Left: Bruker Avance III Ascend 400 MHz NMR Right: Agilent 1200 Series LC with Binary pump.

As per standard with molecules such as these, the use of matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), gas chromatography coupled to mass spectrometry (GC-MS) together with different types of nuclear magnetic resonance (NMR – left panel on **Figure 1.8**) is recognized as an efficient method of decrypting the chemical general structure of several components of a SL mixture. Using these methods tied with previous purification/fractionation such as through liquid chromatography (LC – right panel on **Figure 1.8**) using reverse phase C18 columns or thin-layer chromatography (TLC) can prove even better. Fourier transform infrared spectroscopy (FT-IR), if possible, on previously isolated fractions of SL can also lead to a thorough report on the structural variety of a mixture.

Furthermore, when the objective of the work is to propose an industrial use for biosurfactants such as these, authors are pressed to perform assays of surface tension and emulsification so as to lastly conclude if the obtained product has better or equivalent surfactant capabilities than synthetic detergents and can be used as biodegradable and cleaner alternatives to these (Claus and Van Bogaert, 2017).

1.7 Medicinal, commercial and industrial potential

Many studies have showed up in the last two decades that intended on coming up with creative and novel utilities for biosurfactants, predominantly sophorolipids. All the characteristics mentioned in previous sections of the introduction are what bestow sophorolipids with the title of added-value product. The fact that it can be produced quite easily by several species of non-pathological microorganisms and in large quantities while offering great foaming and emulsion characteristics make it a very promising candidate for industrial production and commercial venues (Akbari et al., 2018) and their proven anti-tumoral and antimicrobial activity also makes them a target molecule for biomedicine and bionanotechnology approaches (Callaghan et al., 2016; Naughton et al., 2019).

Although the potential of these biomolecules has been proven, for some reason, only a few companies, such as Evonik and Holiferm, are giving them commercial applications, but none appear to be industrial scale operations. If searching around for patents with sophorolipids as a search key, one will find that some synthesis processes and medicine-related aspects (such as antifungal and even some virucidal appliances) are either registered, archived or still in the course of being validated. This is particularly odd for a biomolecule that has been known to exist since at least 1961 (Gorin et al., 1961) and has received an affluence of knowledge in the last two decades. At an academic level, some studies have appeared with the intention of upscaling the production process to pilot plant scale. (Peñalver, 2017)

With all this in mind, perhaps in the next few years we might see an exponential increase of industrial scale products making use of these very interesting biosurfactants.

1.8 Objectives of the work

Initially, this work had as a starting point generating proof-of-concept for the repurposing of WGP as a microbial substrate in the production of sophorolipids, glycolipid molecules with good tensioactive properties and of high added-value. These lipids are known to be secondary metabolites produced by yeast species such as *Starmerella bombicola*, preferably from a mixture of carbon sources containing both free sugars and oils.

Likewise, the idea to come up with a clean, sustainable and low-cost process for the repurposing of this agro-industrial residue was key for development of a considered successful strategy. Supercritical carbon dioxide extraction was the chosen method to try and retrieve the oils present in WGP and a simple water extraction at low temperatures was the method intended to obtain the WGP sugar-rich solutions from the abovementioned residue, needed as main hydrophilic substrate.

After this was done, the next step would be to optimize the yields of both WGP extracts with biotransformation potential, to compose an optimal hydrophilic (WGP sugar-rich liquor) and hydrophobic (WGP oils) carbon source-based culture medium on which a fructophilic and oleaginous yeast, *Starmerella bombicola*, could thrive and produce the mentioned added value biosurfactant with

fairly decent yields. These sophorolipids are commonly known to only be produced by ascomycetous and non-pathogenic yeast strains of the *Starmerella* clade, *Candida apicola*, Candida *batistae* and *Candida floricola* as well as *Rhodorotula bogoriensis* and *Wickerhamiella domercqiae* (Konishi et al., 2016). The performance of the yeast cells in terms of biomass quantity and sophorolipid production would then be evaluated, both in media containing only free sugars and media composed of a mixture of both WGP extracted sugars and oils.

Those sophorolipids, produced in different culture media, were then to be identified, purified and characterized by LC-MS, ^{1}H-NMR and compared to other synthetic surfactants by studying their surface tension parameters.

2 Materials and methods

2.1 White grape pomace

WGP used throughout the experiment was provided by a Portuguese wine producer after the wine making process and was kept in plastic bags, refrigerated at -20 °C, to avoid microbial fermentation. For further experiments, all WGP used was first dried in an oven at 60 °C overnight, to remove maximum water content whilst maintaining all other compounds of interest unscathed. WGP mass was measured before and after drying overnight at 90 °C to determine water content gravimetrically.

WGP dried at 60 °C was submitted to grinding in a regular kitchen grinder for supercritical fluid extraction experiments, and in an IKA Tube-Mill miller at 10 000 rpm for 10 seconds to circa 1 mm for all experiments involving lipid extraction by Soxhlet. When not used, WGP was stored at -20 °C.

2.2 WGP partial chemical characterization

2.2.1 Lipid extraction

The Soxhlet extraction method was the technique of reference to compare yields to the $scCO_2$ experiments.

2.00 g of grinded WGP solid were placed inside a package made of 0.22 µm pore size filter paper and weighed. The package was inserted in the Soxhlet extractor and subjected to a volume of 65 mL of solvent grade n-hexane (Carlo Erba, pure). Full extraction was obtained after 33 cycles.

The residue was left to dry at 40 °C overnight to remove traces of n-hexane. This was done to ensure a stable measurement of weight for the solid residue is obtained, on an analytical scale.

The hexane-oil extract was taken to a rotary evaporator, at 45 °C until all the solvent was evaporated, aided by gaseous nitrogen flux if needed after which only the dry WGP oil residue remained.

At this point, both the flask containing the dried WGP oil and the remaining defatted WGP residue were weighed. The obtained measurements were then used to calculate a weight/weight percentage (wt. %) that considers compounds soluble in hexane. The oils obtained were stored at -20 °C.

2.2.2 Supercritical CO_2 extraction: conditions and apparatus

100 g of WGP grinded to medium to large particle size were added to the extractor, mixed with glass beads to facilitate CO_2 diffusion and dispersion. Cotton discs were placed at both ends of the extractor to avoid loss of WGP.

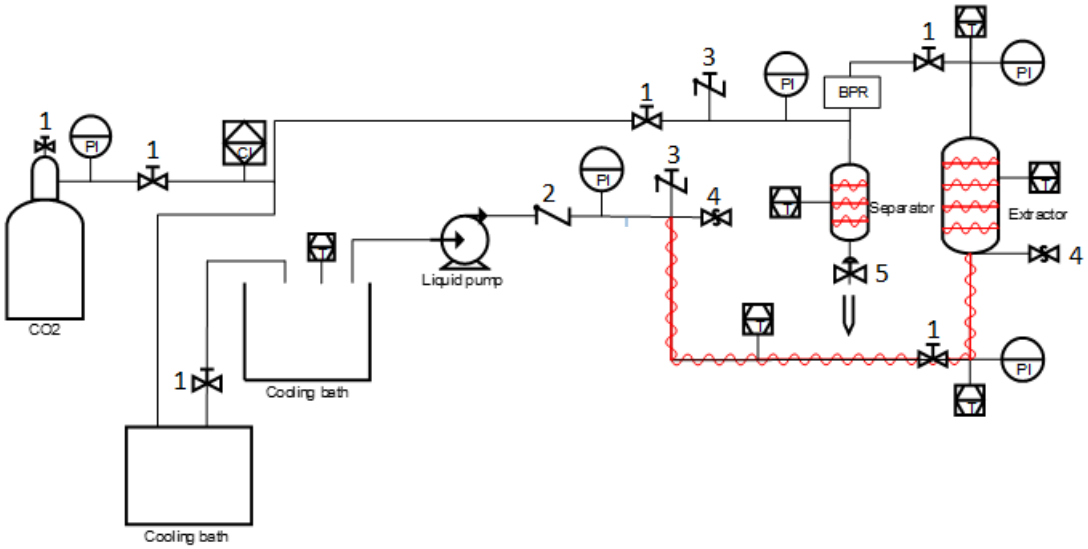

Figure 2.1 – Schematics of supercritical CO2 extraction unit: 1 – Needle valve; 2 – Check valve; 3 – Safety rupture disc; 4 - Relief valve; 5 – Sample collection valve. BPR – Back pressure regulator. Thermocouples are positioned together with the heating stripes, upstream and downstream of the extractor and on the second cooling bath.

Four experiments were performed, two at an extraction temperature of 40 °C and two at an extraction temperature of 45 °C. Separator temperature was 50 °C. Initial CO_2 pressure was 6 MPa until compressed by the liquid pump, identified on **Figure 2.1**. Pressure was 50 MPa with an average CO_2 flow rate of 13 g CO_2/min for all experiments.

2.2.3 Lipid profile determination: GC analysis

The WGP oil extract was submitted to a transesterification reaction with methanol (Honeywell, pure) at a methanol 24:1 water molar ratio, using n-hexane as solvent (2 mL). The oil concentration was 20 mg/mL. As such, a 5 mL vial was used in which 40 mg of oil were placed. 100 mg of lipase immobilized on immobead 150 from *Candida rugosa* (Sigma Aldrich) were added to the mixture oil and the reaction was initiated by addition of 40 µL of methanol. The mixture was left in a shake incubator at 300 rpm, 37 °C for 48 h.

For the GC-FID analysis (Thermo Scientific Trace GC Ultra, SSL injector), methyl heptadecanoate (Honeywell Fluka, ≥99 %) was used as internal standard.

GC-FID analysis program began at 90 °C, rising to 110 °C in 30 seconds followed by a heating ramp at 4 °C/min up to 230 °C and finished after 5 mins at this temperature. Flame ionizing detector (FID) was at 280 °C. All samples were tested in duplicates.

2.2.4 Free sugar extraction

Pedras et al., (2017) reported that this WGP extract had a 40 wt. % of soluble (free) carbohydrates. In current work, extraction of WGP free sugar extract was done by simple water extraction. To confirm the free carbohydrate content given by Pedras et al. and determine the relative amounts of glucose and fructose, experiments were performed in which 200 mL dH_2O were added to a 1 L Schott flask containing 10 g of the dry and defatted WGP. The flask was agitated via magnetic stirring at 800 rpm for 1 h, at 35 °C, resulting in a WGP free-carbohydrate-rich liquid. The liquid was twice centrifuged at around 9 700 x g for 30 minutes and the supernatant was retrieved. Samples were sent for HPLC analysis.

For yeast growth assays, the amounts of water and WGP were adjusted so as to obtain a solution with a target concentration of 50 g/L of carbohydrates. To try and obtain a target concentration of 100 g/L SCS, two different water extractions were performed, each for 50 g/L SCS. One of the solutions was lyophilized for 48 h (Christ Alpha 1-4, Braun Biotec International). The resultant dry WGP free sugar extract was then weighed, and the correct amount was added to the 50 g/L WGP free sugar solution.

2.2.5 Free sugar determination: HPLC method

A sample of 1 mL was retrieved from the WGP free carbohydrate solution, centrifuged at around 10 000 x g for 5 mins and then filtered using a 1 mL syringe with a 0.22 µm nylon filter attached to the tip and pressed into a HPLC vial, to remove possible solids.

The samples were then injected into a Dionex P580 HPLC equipped with a 300 x 7.8mm and 9 µm pore size Aminex HPX-87P column (Bio-Rad) coupled with an IR differential refractometer LKB, model 2142. The mobile phase was sterile dH_2O with a small percentage of sodium azide and flow rate was kept steady at 0.6 mL/min. Temperature was kept steady at 70 °C. Calibration curves, peak integration and result analysis were automatically obtained with the Chromeleon™ Chromatography Data System Software by Thermo Scientific and results are expressed in g/L.

2.2.6 Free carbohydrate extract elemental analysis

The elemental NCHS analysis of the WGP free carbohydrate extract was performed at Laboratório de Análises, REQUIMTE-LAQV, FCT-UNL. Results were analyzed by the same unit.

2.3 Yeast growth experiments

2.3.1 Yeast strain maintenance

Starmerella bombicola PYCC 5882 was originally from the Portuguese Yeast Culture Collection (PYCC), Caparica, Portugal. The yeast strain was maintained at 4 °C, in YPD medium composed of 20 g/L glucose, 10 g/L bacterial peptone, 10 g/L YE and 20 g/L agar.

2.3.2 Yeast hydrophilic carbon source growth media

Experiments concerning yeast growth on sugars as carbon source were performed in different medium component concentrations, two with glucose and fructose and the third one with WGP free carbohydrates liquor.

The first control experiment, control A, was performed in a medium containing 25 g/L of glucose and 25 g/L of fructose, supplemented with 5 g/L YE, achieving a Sg/YE ratio of 10. Culture initial volume was 400 mL.

The second control experiment, control B, was performed in a growth medium of the same glucose and fructose concentrations as control A but with 2.5 g/L YE, to get a Sg/YE ratio of 20. Culture initial volume was 400 mL.

The experiment utilizing only the WGP SCS as hydrophilic carbon source, experiment B0, was set at a concentration of 40 g/L sugar (mainly glucose and fructose) and YE 2 g/L, to maintain the same Sg/YE ratio of 20 as control B. Culture initial volume was 300 mL, due to WGP extraction volume limitations.

Glucose and fructose stock solutions were vacuum filtered using a 0.22 µm PES membrane filter. All other components, including SCS, were previously sterilized via autoclave at 121 °C for 15 mins.

2.3.3 Hydrophilic and hydrophobic carbon source growth media

For experiments utilizing both hydrophilic and hydrophobic carbon sources, were supplemented at 24 h with 50 g/L WGP extracted oils, hereafter named oil carbon source (OCS) in all growth experiments. Also, several different concentrations of hydrophilic carbon sources were used while YE concentration was maintained at 2.5 g/L for all experiments. Culture volumes were reduced to 200 mL, to facilitate handling.

- Experiments A1 and A2, 50 g/L and 82.5 g/L glucose + fructose, with OCS supplementation at 24 h;

- Experiments B1 and B2, 75.7 g/L and 97.6 g/L SCS respectively, with OCS supplementation at 24h, from this point onwards called WGP media.

2.3.4 Yeast inoculum and growth

A 10 or 20 mL pre-inoculum was prepared in a 50 mL or 100 mL Erlenmeyer flask, respectively, and incubated overnight at 30 °C in an incubator with orbital shaking set at 200 rpm.

A small volume of pre-inoculum was transferred to the culture medium. This small volume of pre-inoculum would be corresponding to a media OD at 640nm of 0.2. During the culture, samples were withdrawn every 24 hours unless stated otherwise.

Optical density at 640 nm was only followed closely throughout the whole experiment time in experiments that were not supplemented with OCS.

Medium pH was adjusted to pH=3.5 in growth experiments using WGP media.

2.3.5 Yeast biomass determination

Samples were retrieved into Falcon tubes, daily, in volumes of 5 to 15 mL, depending on culture volumes, always making sure that by the end of the assay the culture had only lost a maximum of 10% of its initial volume, and to allow for a ± 0.1 mg of measurement error on analytical scales.

Samples were centrifuged at 10 000 x g for 5 minutes at 5-8 °C, to help with precipitation of all non-soluble species like biomass and other solid particles; this supernatant was retrieved for other analysis and the pellet used to determine yeast dry weights or otherwise frozen at -20 °C.

For samples needed for SL determination, a wash step with 1x volume of ethyl acetate (Sigma Aldrich, 99.8 %) was performed in order to retrieve molecules that could still be present in the pellet. The wash volume was placed in a flask and stored at -20 °C after centrifugation at 10 000 x g, for 5 minutes, at room temperature.

A wash with distilled H_2O was the final step and again centrifuged in the same conditions as previously, to remove any residual solvent present in the pellet. The supernatant pellet was then put in an oven at 80 °C-100 °C overnight and weighed inside a previously weighed Falcon tube. Weight value was converted into concentration.

Due to the presence of extremely small WGP particles in suspension on WGP SCS media that precipitate together with the pellet, an average weight value of suspension particles that was based on the amount present in the first sample retrieved for each growth curve (0 h sample) was subtracted from all posterior samples.

2.3.6 Sugar and fermentation product determination

HPLC was the technique chosen to determine fermentation sugar levels. The method used for preparation of the samples was the same as previously explained in 2.2.5, for each daily sample. Results were expressed in g/L against standard calibration curves.

2.4 Extraction and quantitative determination of SL

2.4.1 Extraction of SL from culture media

The extraction method used for obtaining SL from the culture media were several solid-liquid separations in separating funnels, using ethyl-acetate.

The procedure was as follows; as an example, a sample volume of 10 mL is considered: a sample of 10 mL from the yeast culture medium was placed in a Falcon tube and centrifuged. The aqueous supernatant was placed in a separating funnel. To the pellet, in the Falcon tube, 10 mL of ethyl acetate were added. After stirring for 3 minutes and centrifugation, the ethyl acetate supernatant was removed from the Falcon tube and added to the separating funnel.

An additional 10 mL of ethyl acetate were then added to the mixture already in the separating funnel. After thorough mixing, the contents of the separating funnel were allowed to settle. SL tend to accumulate on the aqueous-organic interphase. Once phase separation was clearly defined, the aqueous phase was removed carefully and submitted to new extraction with ethyl acetate according to the same procedure. The ethyl acetate phase containing SL was placed in a round-bottomed flask. This step was repeated as many times as necessary to ensure that, at the end of the extraction process, no SL were visible at the interphase or in suspension, when stirred. All ethyl acetate fractions were then combined.

2.4.2 Sophorolipid purification

In samples obtained from fermentation media supplemented with OCS (section 2.3.3), if the presence of oils was still visually noticeable, an additional washing step with several volumes of n-hexane was added, until the resulting organic phase no longer shows a yellowish tone. These volumes of n-hexane/oil mixture were subsequently evaporated in a rotary evaporator at 40 °C. The residual oil obtained, of greenish yellow color, was then weighed to access oil conversion percentages for experiments B1 and B2.

SL residues were then dried under nitrogen and stored at -20 °C and had a dark-brown appearance.

2.4.3 Dry weight determination

After ethyl acetate was removed using a rotary evaporator and washed with n-hexane if needed, as stated in 2.4.1 and 2.4.2 respectively, remaining SL residue was weighed on the round bottomed flask.

Weight measurement was then converted into g/L, g SL/g sugar and g SL/ g biomass.

2.5 Structural and chemical characterization of SL

2.5.1 LC-MS analysis

The LC-MS analysis was performed by Laboratório de Análises, REQUIMTE-LAQV, FCT-UNL. The LC column used was a reverse phase Waters Atlantis dC18 4.6x30 mm, 3 µm pore size equipped into an Agilent 1200 series LC with binary pump and UV-vis detection in tandem with an MS-ESI apparatus (Agilent 6130B Single Quadrupole LC/MS with API-ES source).

The mobile phase consisted of eluent A water-formic acid (99.9 %: 0.1 %) and eluent B acetonitrile-formic acid (99.9 %: 0.1 %), at 35 °C. The elution program used was isocratic 50 % eluent A and 50 % eluent B for 5 min followed by gradient elution up to 60 % eluent B for 20 min then gradient elution up to 100 % eluent B for 20 min and finally an equilibrium time of 10 min. Flow rate was kept steady at 0.4 mL/min with an injection volume of 5 µL of 500 ppm samples.

MS instrument was operated in positive and negative ion mode, ESI capillary voltage was 4 kV with a cone voltage set to 60 V with source temperature of 350 °C. Positive mode results were not considered.

2.5.2 ^{1}H-NMR analysis

The ^{1}H-NMR analysis was performed by NMR Service, REQUIMTE-LAQV, FCT-UNL. The RMN device used was a Bruker Avance III 400 MHz with a QNP probe. The solvent used for sampling was $CDCl_3$.

2.5.3 Critical micelle concentration and minimum surface tension determination

Surface tension measurements were performed on a Sigma 702 (KSV) force tensiometer apparatus using a platinum Du Noüy ring against a water standard tension value of 70.75 mN/m ± 0,05 mN/m. Nine solutions of different SL concentrations were prepared in MilliQ water (130 mg/L, 100 mg/L, 65 mg/L, 40 mg/L, 20 mg/L, 10 mg/L, 2.5 mg/L, 1 mg/ and 0.5 mg/L) and tested independently on their surface tension characteristics. All surface tension average values were obtained via Huh-Mason approximation from measurements performed in triplicate.

Critical micelle concentration (CMC) and minimum surface tension (MST) were calculated considering the relation between SL concentration and acquired values of surface tension for each of the solutions.

3 Results and discussion

3.1 Chemical characterization of WGP

3.1.1 Lipid extracts characterization

The Soxhlet method was used to determine the lipidic content in WGP. Several extractions were performed throughout the work. The oil-hexane solution was yellow in color, intensity of color being directly related to the amount of oil retrieved. The average oil content was 13.2 ± 2.8 wt. %, obtained from seven measurements. Values varied between 9.9 and 13.6 wt. %, the results depending on the homogeneity in seeds of the samples used for lipid determination. The ones visually containing more seeds in the beginning presented a higher percentage in oils, as expected, since seeds are the main contributors to the presence of oils in WGP.

Table 3.1 – Oil content of WGP. Values presented are extraction oil minimum wt.%, maximum wt. % and average yield in wt. % of a set of Soxhlet extractions (n=7) obtained throughout the entire work.

Minimum Oil wt. %	Maximum Oil wt. %	Average Oil wt. %
9.9	13.6	13.2 ± 2.8

GC analysis of the WGP lipid extract presented the expected results for white grape seeds in terms of fatty acid profile, after transesterification of the triacyclglycerols into esters. The fatty acid profile was of 69.1 % ± 0.7 of linoleic acid, 15.0 % ± 1.3 of oleic acid, 11.2 % ± 0.9 of palmitic acid and 8.4 % ± 0.5 of stearic acid (**Figure 3.1**). These results fall well within the interval of results obtained by Juhaimi et al., (2017) whose results determined a range from 47.34 % to 72.91 % for linoleic acid, a range of 13.35 % and 26.30 % for oleic acid, from 7.15 % to 16.06 % for palmitic acid and 2.43 % to 6.55 % for stearic acid for several grape cultivars.

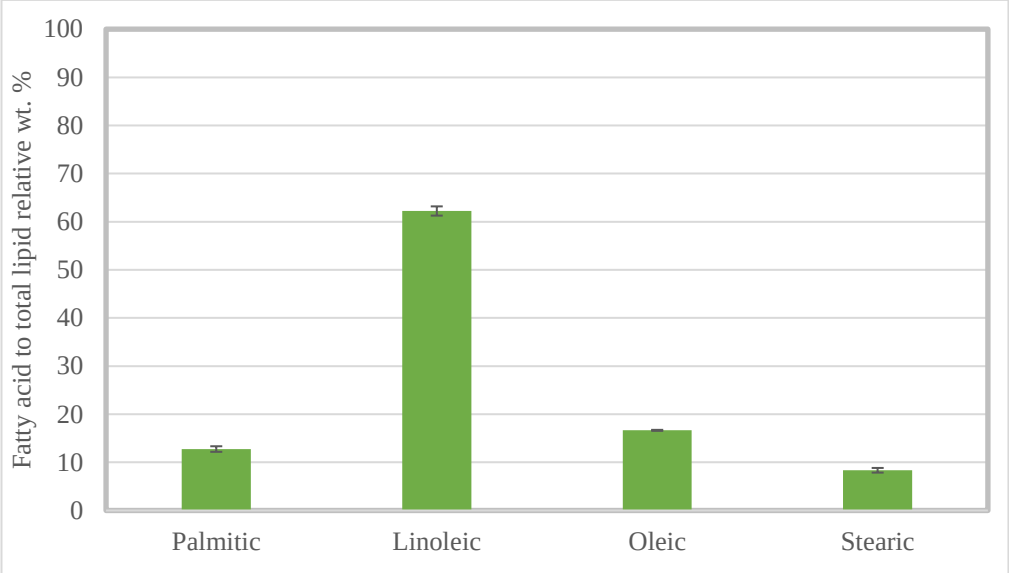

Figure 3.1 - Lipid profile of WGP lipid extracts. Error bars (n=4) represent the standard deviation of results obtained with GC analysis.

One should keep in mind, though, that the geographical location of grape cultivars is a factor to consider when noticing small disparities in nutritional profiles such as these although, in general, the profile of fatty acids should have linoleic acid as being the most present (Tangolar et al., 2009).

According to these results, these oils seem to be adequate for *S. bombicola* to use towards the production of SL as it is rich in long chain fatty acids (C16 and C18) (Claus and Van Bogaert, 2017).

3.1.2 Supercritical CO_2 extraction kinetics

In order to ensure a green method for the extraction of WGP oils, supercritical fluid extraction was chosen as a possibility, using $scCO_2$ as the lone solvent. Pressure was kept at 50 MPa throughout all experiments while initial temperature was 40 °C for the first two extractions. Later, two other extractions were performed at a temperature of 45 °C. Total CO_2 expended mass and flow rate were kept constant at 13 g/min.

Two kinetic curves were plotted (**Figure 3.2**), one kinetic curve obtained as an average of two experiments at 40 °C and a second kinetic curve from two other experiments at 45 °C, as the extraction behavior was similar when operating under the same conditions of temperature.

At 45 °C, it is noticeable that the extraction was more efficient in terms of time expended. According to **Figure 3.2**, the final extraction baseline is achieved in around 50 minutes, the yield in wt.% does not vary much more and retains a value around 4.38 wt.% up to the last point of extraction, at 120 minutes. The kinetics of the 50 MPa, 40 °C extraction takes longer to reach this standpoint,

only arriving at the maximum baseline after 90 minutes of run. The fact that both experiments reached the same value, although at different speeds, implies that at these conditions either due to the WGP particle size or the temperature at which the extraction is occurring, 4.38 wt.% is the maximum yield obtained. Pressure around 50 MPa should not be the problem according to literature (Duba and Fiori, 2016), various authors using such pressure and stating it to be the best pressure on which to obtain the highest yields.

The problem should reside on particle size. In fact, particle size of WGP on $scCO_2$ extraction experiments was different from the particle size used for Soxhlet extractions. For $scCO_2$ extraction experiments, WGP was ground with a regular kitchen counter blender instead of the laboratory grade miller used for most Soxhlet extractions. This perhaps reduced the performance of the extraction unit, explained by the fact that CO_2 couldn't diffuse properly into the large particles (José and Uquiche, 2002) which in turn led to the yield percentage of the extraction to not come near the yield average percentage values obtained through Soxhlet extractions, as these were done with a finer grind of WGP, with particles ranging from 650 to 1000 µm.

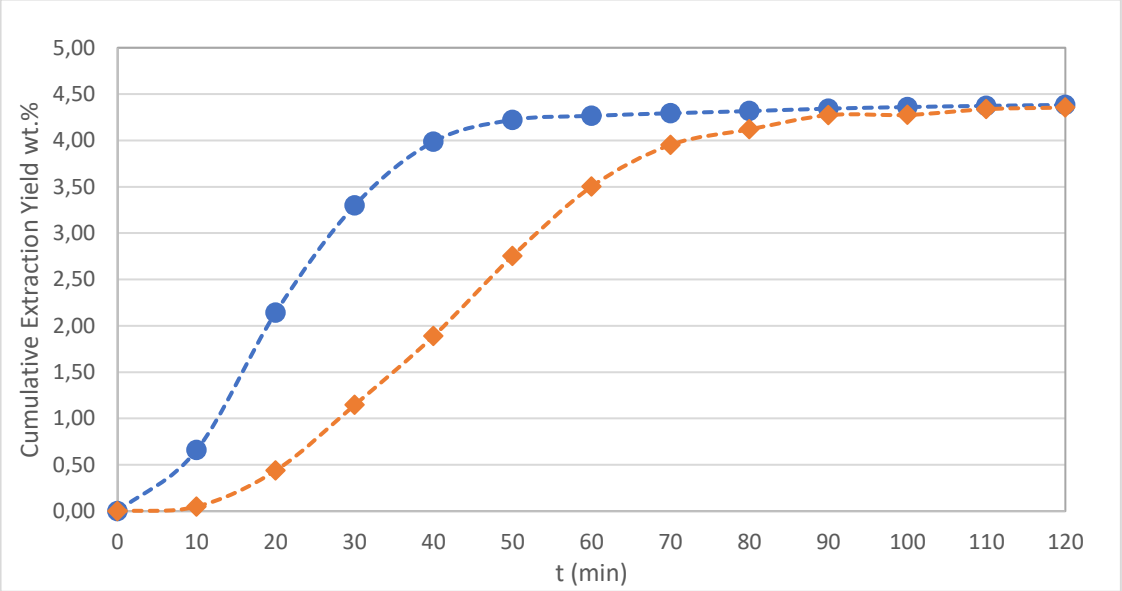

Figure 3.2 – The two representative kinetic curves plotted at 500 MPa pressure and temperatures 40 °C in orange diamonds and 45 °C in blue circles, from two independent experiments for each temperature. CO2 flow rate was kept around 13 g/min for all experiments.

Due to the much higher yield of extraction obtained with the Soxhlet apparatus when compared with this method, Soxhlet extraction was chosen as the preferred method to obtain lipid extracts from WGP, which would later be used to supplement yeast growth cultures with the purpose of improving SL yields greatly. To keep the process as green as possible, n-hexane would always be recovered and used as many times as possible to obtain lipid extracts that had the same purpose of usage, in future experiments.

3.2 Free carbohydrate extracts characterization

The free carbohydrate extraction was performed to oil-free WGP, to facilitate dispersion of WGP particles in the water.

Table 3.2 – Sugar composition of WGP sugar extracts in wt. % for n=8.

	Average g/100 g dry WGP
Glucose	20.5 ± 1.2
Fructose	22.2 ± 3.3
Total	42.7

Free sugars, more specifically glucose and fructose, are the main bulk of WGP, rising up to 42.7 wt.% in content (**Table 3.2**). The reason behind the presence of such a large amount of free sugars is that WGP does not undergo fermentation and thus retains the entirety of the free sugars. In general, glucose and fructose tend to be present in equal amounts in white grapes (Amerine, M A; Thoukis, 1958) even though other free sugars such as fucose and sucrose might also present, but in residual quantities; fucose going as far as 1.5 wt.% and sucrose around 0.2 wt.%, as previously determined by Pedras (2015). A total free carbohydrates value of 42.7 wt. %, was obtained and is considered in agreement with previously recorded experiments of the research group. Structural sugars were not recovered from WGP as temperatures used during the extractions throughout this work were not sufficient to hydrolyze them.

3.3 Growth experiments towards the production of SL

3.3.1 Hydrophilic carbon source media

3.3.1.1 Commercial sugars media

In order to better understand the behavior of the yeast used throughout this work and how its growth would behave across the experimental time periods, some control experiments using only commercial glucose and fructose and YE as nitrogen supplement for growth were done.

Control A (**Figure 3.3**) was performed with an initial glucose + fructose concentration of approximately 48 g/L (21 g/L of glucose and 27 g/L of fructose) and a supplement of 5 g/L of YE. The culture was followed for 144h and maximum biomass attained was 8.4 g/L, at 144 h.

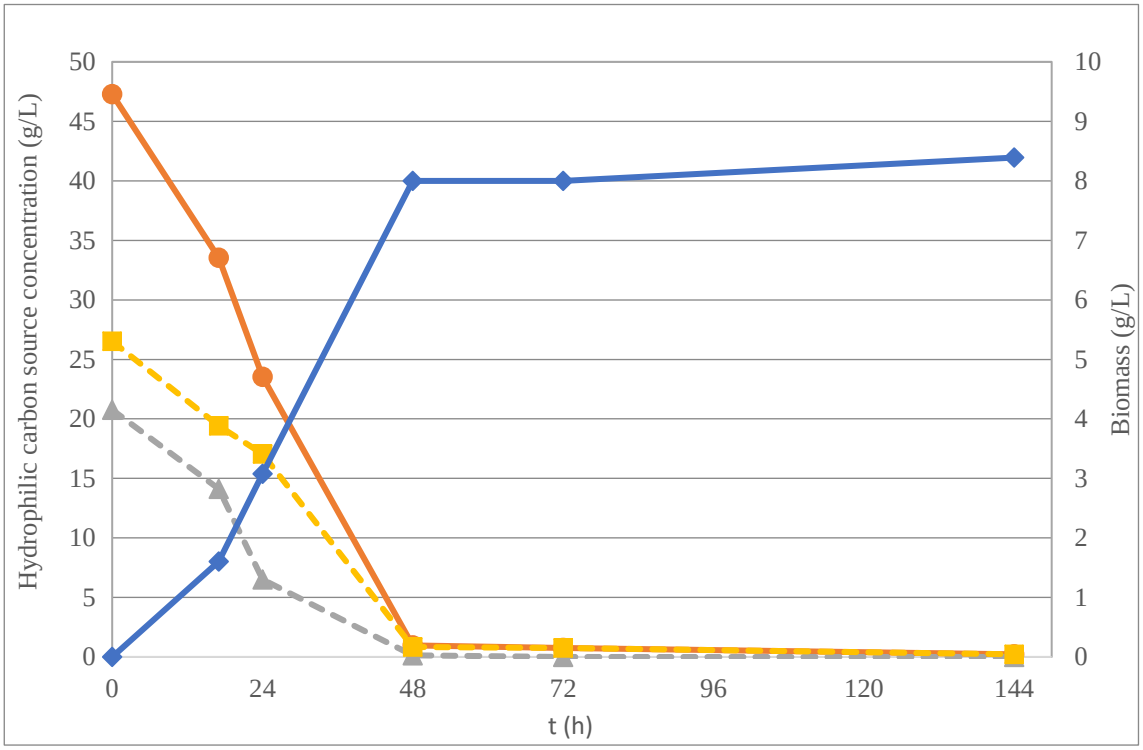

Figure 3.3 - Graph depicting control A, *S. bombicola* growth and sugar consumption throughout 144 h when medium conditions were 48 g/L glucose + fructose and 5 g/L YE. The graph is represented as sugar concentration in medium (g/L) and biomass (g/L) through time (h). Represented by squares – fructose; triangles – glucose; circles - glucose and fructose; diamonds – biomass.

When it comes to *S. bombicola*, SL production is known to start at the beginning of the stationary phase and prolongs itself for as long as the cells can sustain its production, by *de novo* synthesis in the absence of readily available hydrophobic resources such as fatty acids or long/medium chain hydrocarbon molecules (e.g.1,12-dodecanediol; 12-hydroxydodecanoic acid, etc.), or by using the fatty acids present in the surrounding medium to integrate in their respective

SL structures (Van Bogaert, 2008). Yields for SL built from *de novo* synthesis tend to be much lower than those produced when the yeast culture media is actively supplemented with hydrophobic carbon sources (Davila et al., 1994).

This control was therefor also built to guarantee that the yeast culture utilized still possessed the usual metabolic functions, offering an abundance of nitrogen sources from which the yeast can produce proteins and DNA for its reproduction and metabolic needs, and to determine the SL production by *S. bombicola* PYCC 5882 when growing solely on sugar. To that avail, a daily SL production profile was traced (**Figure 3.4**).

The yeast behaved as expected because the production of sophorolipids, in the absence of a hydrophobic carbon source, began precisely at the same time as biomass measurements indicated the yeast was entering the stationary phase of growth, as stated by kinetic model studies that analyzed the behavior of *S. bombicola* metabolism over its several stages of growth (Alcon et al., 2004). The ratio of 50 g/L hydrophilic substrates over 5 g/L yeast extract represents a theoretical sugar to YE ratio (henceforth called sugar to nitrogen Sg/YE ratio, for simplification of discourse) of 10.

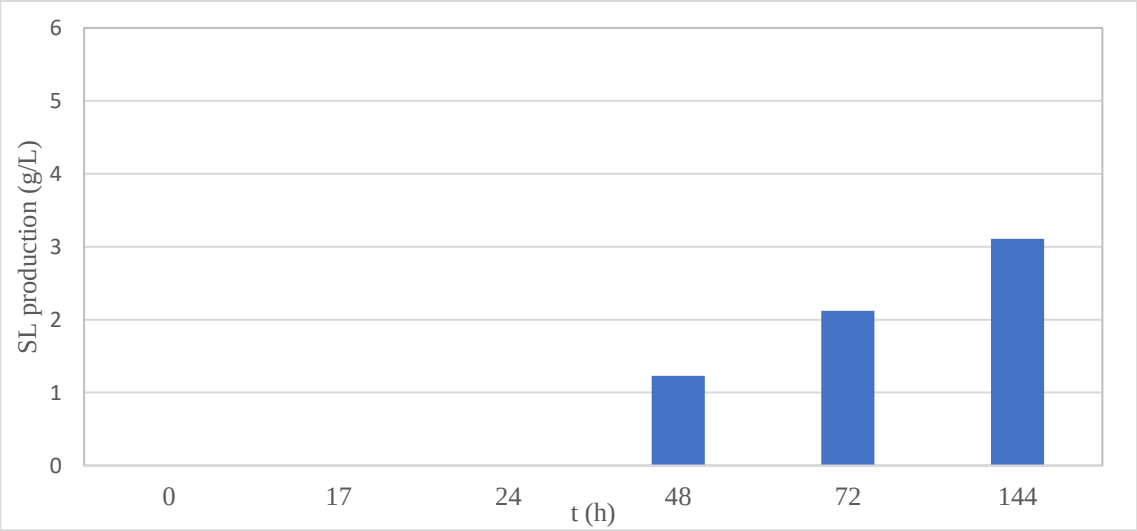

Figure 3.4 - SL production profile of *S. bombicola* when only in contact with hydrophilic carbon sources. Bar graph represents SL concentration (g/L) of control A, represented in **Figure 3.3**. The production starts when yeast cells enter the stationary phase as long as they can maintain surviving conditions afterwards.

This factor was taken into consideration for the following experiment, control B. This new control experiment was done because the available free sugars in control A were expired very quickly from the fermentation media and the yeast cells produced a small amount of SL. This control would then be used to see how the yeast would behave in a larger Sg/YE ratio of 20, by lowering YE concentration to 2.5 g/L and maintaining the same concentration of both sugars with the intention of understanding if glucose and fructose were still present in the medium after 72 h for the yeast to have some substrate with which to work towards the production of SL.

The results of control B, represented in **Figure 3.5**, show that with a larger Sg/YE ratio of 20, *S. bombicola* grows slightly less in terms of biomass (6.3 g/L biomass in comparison to 8.0 g/L at 72 h) while consuming sugars at a slower pace, reaching 72 h with glucose still present. One can also notice that fructose seems to be consumed at a faster rate than glucose as expected for *S. bombicola*, a known trait in this yeast (Gonçaves et al., 2018). The fructophily observed in this yeast species offers new prospects for investigation as it is an interesting trait to explore when creating efficient media for *S. bombicola* to thrive and grow and where it can produce even higher quantities of SL. The yeast cells showed less growth perhaps because of the lower concentration of nitrogen available and, consequently, they might have consumed less free sugars because there was less biomass produced.

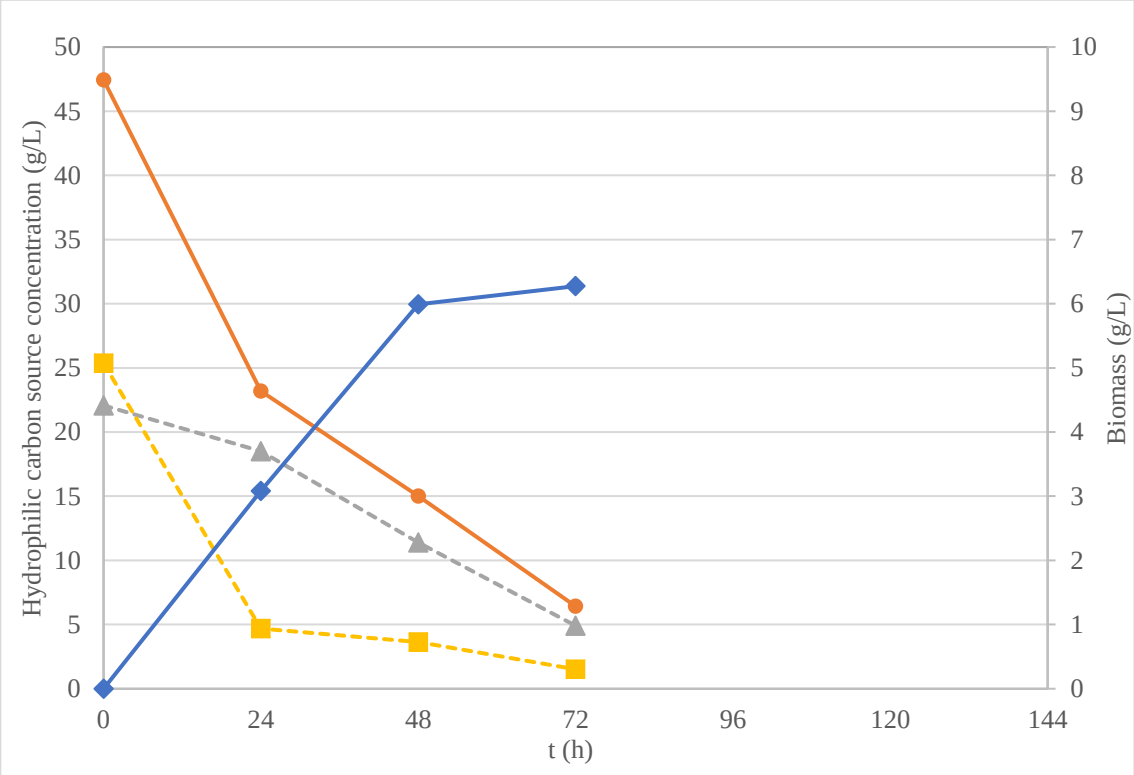

Figure 3.5 – Graph depicting experiment control B with biomass concentration (g/L) as well as hydrophilic carbon source consumption (g/L), up to 72 h, when culture growth conditions revolve on a larger initial sugar to yeast extract ratio (47 g/L glucose and fructose to 2.5 g/L YE, Sg/YE ratio of 20). Squares – fructose; triangles – glucose; circles - glucose and fructose; diamonds – biomass.

Additionally, to make for a better comparison between both experiments, sugar consumption rate (g/L.h) for the exponential phase (up to 48 h) was calculated and can be seen in **Table 3.3**, together with acquired values in g/L for glucose, fructose and biomass.

Table 3.3 –Glucose and fructose concentration (g/L) and biomass (g/L) at 72 h. Sugar consumption rate calculated from the sugar consumption curve of control A and control B up to 48 h.

	Control A	Control B
Glucose (g/L, 72 h)	0.14	11.39
Fructose (g/L, 72 h)	0.83	3.63
Biomass (g/L, 72 h)	8.0	6.3
Sugar consumption rate (g/L.h)	0.98	0.67

Evaluating the results obtained, the biological behavior of *S. bombicola* was in accordance with literature (De Graeve et al., 2018) and with the previous control experiment. With all this in mind, a third experiment was planned, this time focusing on an alternative hydrophilic carbon source, WGP free sugars.

3.3.1.2 WGP SCS media

This fundamental experiment was put in place to ensure that future experiments, relying solely on WGP extracts would be efficient in the production of SL and would not influence in any way the regular metabolic performance of *S. bombicola*. Also, attention was put on possible nitrogen presence in the WGP SCS solution. To certify that nitrogen was not present in the WGP SCS extract, NHCS elemental analysis was performed on lyophilized WGP free sugars. Results indicated a negligible presence of nitrogen; they can be seen on **Table A 1**. Here on out named B0, this experiment showed promising results (**Figure 3.6**).

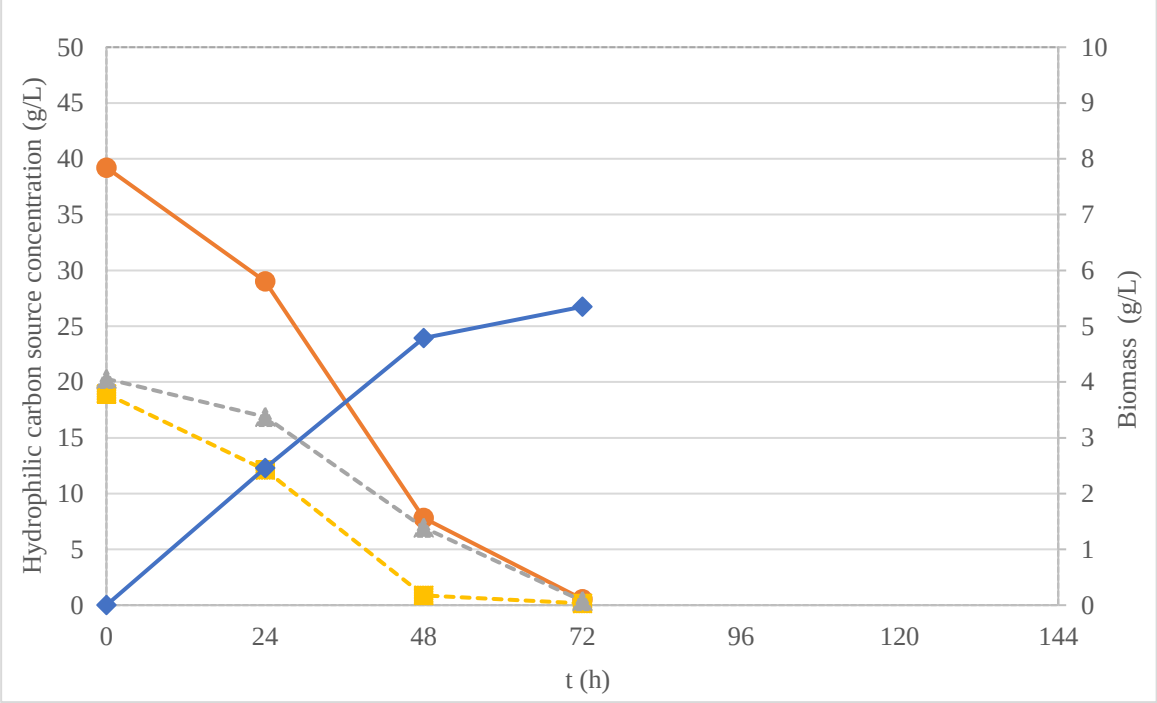

Figure 3.6 - Graph depicting experiment B0, medium composed of 39 g/L SCS and 2 g/L YE. Primary Y axis accesses sugar consumption (g/L) while secondary Y axis is relative to biomass (g/L), both in relation with time (h). Squares – fructose; triangles – glucose; circles - glucose and fructose; diamonds – biomass.

The behavior was as expected for *S. bombicola* when grown in a medium with hydrophilic carbon sources only, serving as proof-of-concept that free monosaccharides extracted from WGP would be fitting to produce low-cost media for *S. bombicola*. Also, a SL production of 2.7 g/L was seen after 72 h. A summary table of all SL production yields obtained for this section of the work can be examined in **Table 3.4**.

Table 3.4 - Summary table of all SL yields and production values obtained at 72 h with the three control experiments expressed in g/g biomass, g/g sugar and g/L, alongside the Sg/YE ratio.

Experiments	SL g/L	g SL/g sugar	g SL/g biomass	Sg/YE
Control A (72 h)	2.1	0.045	0.370	10
Control B	3.4	0.067	0.512	20
Experiment B0	2.7	0.055	0.460	20

It is perceptible that experiments where the Sg/YE ratio was maintained at 20 lead to a slightly greater SL production though one should take notice that the initial concentration of monosaccharides is also important as, at 72h, all carbon sources are clearly expensed on assay B0 whereas in control B there are still some monosaccharides available for *S. bombicola* to use. Control A, because of the smaller Sg/YE ratio of 10, also had no monosaccharides left at 72 h.

Unfortunately, and despite outcomes being similar to control B, assay B0 had a setback: the WGP water extraction to obtain a liquor solution of 50 g/L SCS (glucose and fructose of WGP origin) yielded only a concentration of 39 g/L SCS. Due to this preponderant factor, YE concentration had to be reduced to 2 g/L, in order to respect the Sg/YE factor of 20 used in control B. This slightly lower concentration of YE and monosaccharides, even while maintaining the same Sg/YE ratio as in control B, might have influenced the growth of the cells because there was almost no sugar remaining in the medium at 72 h, unlike what happened on control B. Moreover, biomass at 72 h was also slightly lower to the above mentioned experiment, going up to only 5.4 g/L.

The combined results of these three control experiments with just hydrophilic carbon sources as growth and production substrate allowed for the preparation of new experiments, this time all of them containing supplementation with oil carbon sources (OCS).

3.3.2 Hydrophilic and hydrophobic carbon source media

After careful planning and analysis of results obtained from control experiments, four other growth experiments were proposed. Out of these four, two would be performed with commercial glucose and fructose while the other two would be using WGP sugar carbon sources (SCS) as their main substrate for growth. Additionally, all of them were to be supplemented with WGP oil carbon sources (OCS). This time, however, a pH of 3.5 was set as pH reference value to which all cultures would be adjusted to, if needed, stated in literature to be optimal for SL production (Geys et al., 2018).

The thought behind this was that maybe by increasing the Sg/YE ratio whilst allowing for enough nitrogen to be available in the growth media for substantial biomass production would result in increased yields in SL throughout the board, amplified by the supplementation with oils that according to literature (Daverey and Pakshirajan, 2009; Ul et al., 2018) also exponentially increase SL production.

3.3.2.1 Commercial sugars and WGP oil carbon source fermentation media

The first experiment setup was based on a medium containing a combined concentration of 50 g/L of glucose + fructose and 2.5 g/L YE (Sg/YE ratio of 20), with supplementation of 50 g/L OCS when the culture reached 24 h, named experiment A1.

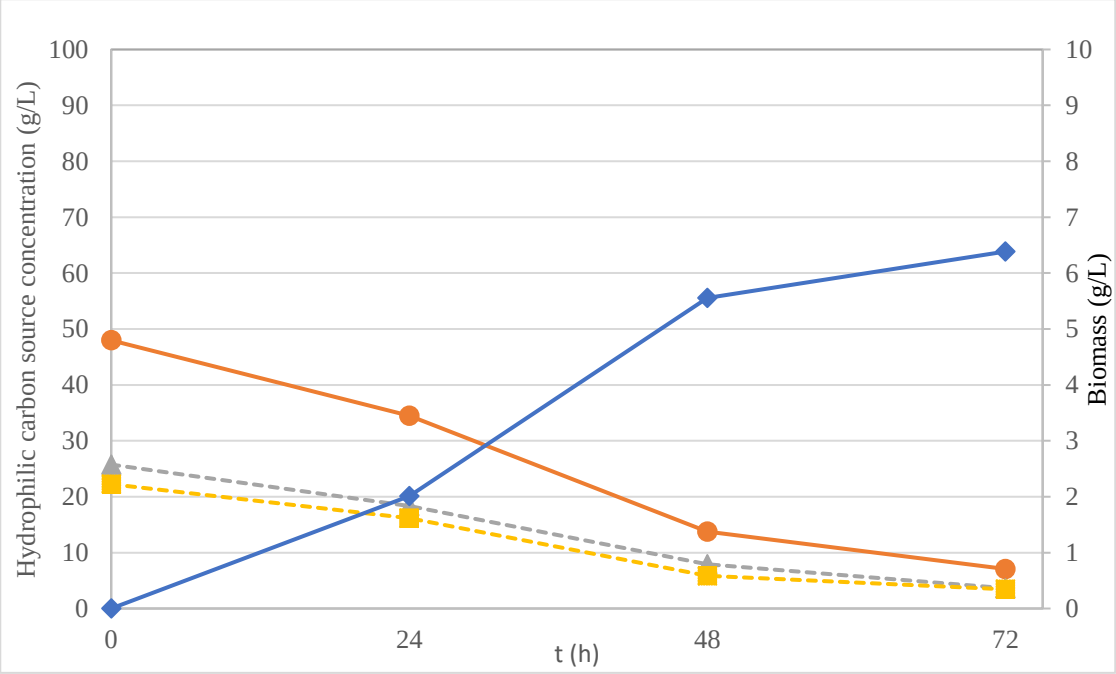

Figure 3.7 - Graph depicting experiment A1. Hydrophilic carbon source initial concentration is 48.0 g/L and final biomass is 6.4 g/L after 72 h. Squares – fructose; triangles – glucose; circles - glucose and fructose; diamonds – biomass.

Monosaccharide consumption and biomass produced in this experiment are represented in **Figure 3.7**. What one would hope to see with this experiment in terms of biomass was precisely the same behavior as in control B. Monosaccharide concentrations in the medium are practically the same and the concentration of nitrogen source, YE, is still 2.5 g/L but OCS were added to the medium after 24 h. The only expected difference for this experiment should be the higher production of SL at the end of the growth curve, 72 h. Several authors mention that the presence of hydrophobic carbon sources in growth medium for *S. bombicola* increases SL production several times fold (Peñalver, 2017).

Appearing as a more suitable comparison to control B (**Figure 3.5**), in this experiment *S. bombicola* seems to adapt just fine to the presence of OCS, with no visual difference in biomass production or monosaccharide consumption, reaching a final biomass concentration on par with control B, at 6.39 g/L. The OCS supplementation created a necessity to wash the SL properly after obtaining them from the fermentation media since not all oils were consumed by the 72 h mark. SL yields for experiment A1 after removal of the residual oil are displayed in **Table 3.5**.

Immediately, the difference between the controls and this experiment is noticeable. Across the board, SL yields for A1 are far superior to those obtained in control B, the best control in terms of conditions and SL production. Even at 48 h, the production is already twice that of the SL yields obtained in control B, after 72 h. This experiment served as a confirmation that WGP oil extracts can safely be used to produce increased levels of SL (Casas and García-Ochoa, 1999; Huaimin et al., 2018; Van Bogaert, 2008).

Table 3.5 - Table with production of SL in g/L and yields in g/g sugar and g/g biomass, for experiment A1, at 48 h and 72 h.

Time	SL g/L	g SL/g sugar	g SL /g biomass
48 h	6.8	0.20	1.22
72 h	10.6	0.26	1.67

A second supplementation experiment, A2, was prepared with a slightly higher concentration of hydrophilic carbon sources, this time in a medium with 82.5 g/L of glucose and fructose (Sg/YE ratio of 33) while attempting to prolong the period of the experiment to 168 h, in order to try and allow for the yeast cells to consume all the OCS that could still be present. Results are displayed in **Figure 3.8**.

The results obtained with this experiment were slightly different from expected. Biomass at 72 h was not on par with experiment A1, actually coming up short, at 5.4 g/L when compared with the 6.4 g/L obtained in A1. This was considered part of the experimental error as no exact duplicates of the cultures were done.

SL calculated production and yields (**Table 3.6**) were somewhat lower at 72 h, unlike what would be expected as a larger quantity of monosaccharides was initially offered in the medium, when comparing with experiment A1. However, at 72 h, the disparity in monosaccharide concentrations was obvious (A2 at 72 h had 6.1 g/L of glucose and 13.3 g/L of fructose still, while A1 had only 3.6 g/L and 3.4 g/L respectively). Maybe, had we prolonged the time for growth of the A1 culture to 168 h, SL production wouldn't reach the level of production attained in A2, due to the lack of readily available glucose for successful integration into the SL sophorose moieties after 72 h.

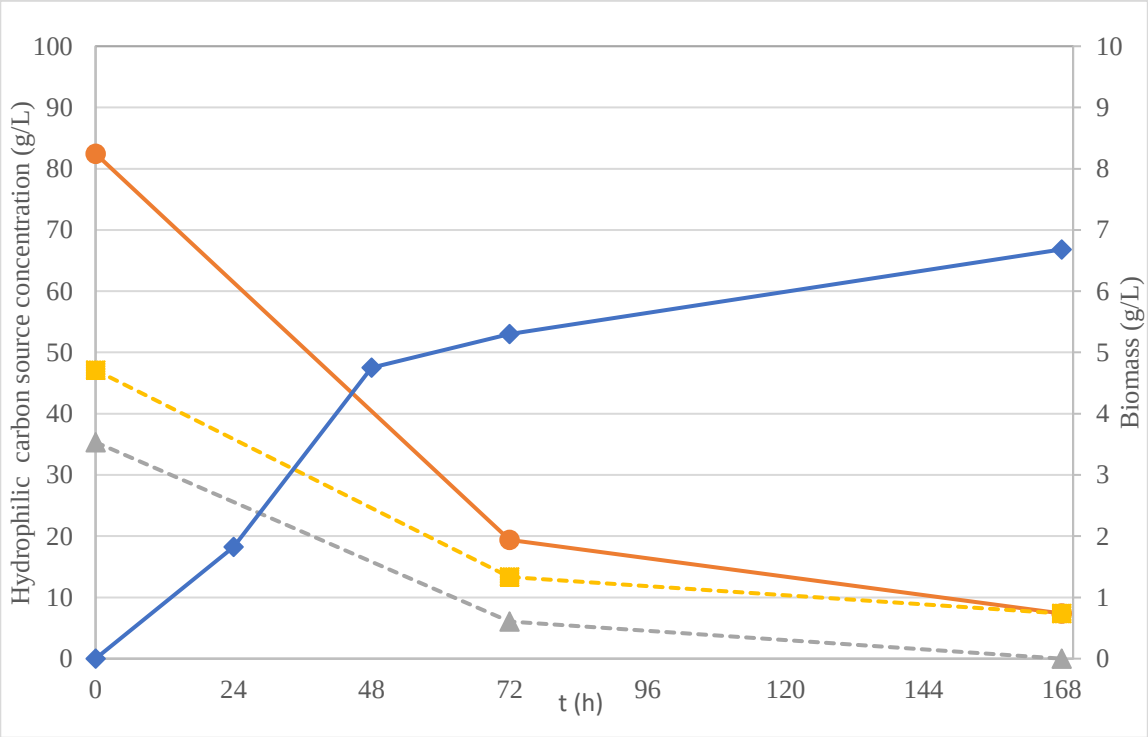

Figure 3.8 - Graph depicting experiment A2. Hydrophilic carbon source initial concentration is 82.5 g/L, read on the primary Y axis and final biomass is 6.7 g/L after 168 h, read on the secondary Y axis. Squares – fructose; triangles – glucose; circles - glucose and fructose; diamonds – biomass.

This slightly different behavior between A1 and A2 might be related to the levels of monosaccharides being close to being expended at 72 h, on experiment A1. This in turn could be displayed as an increased amount of SL produced at 72 h when in comparison with the A2 experiment.

Table 3.6 - Table with SL production in g/L as well as yields in g SL/g sugar and g SL/g biomass, for experiment A2, at 72 h and 168 h.

Time	SL g/L	g SL /g sugar	g SL/g biomass
72 h	5.3	0.18	1.00
168 h	21.1	0.60	3.15

Since the whole objective of this work was to use a low-cost medium based on WGP extracts to produce as much SL as possible, the following experiments were also prepared to go for 168 h.

This was done to ensure that as less oils were left as possible in the media while delivering better yields in SL.

3.3.2.2 WGP sugar carbon source and oil carbon source supplementation media

After the extensive work with mixed media up to this point, two final growth experiments were proposed, using fully WGP-based media.

Two WGP media were prepared, one containing 75.7 g/L of SCS and another one containing 97.6 g/L of SCS. Both were supplemented with 50 g/L OCS at 24 h and had an initial concentration of 2.5 g/L of YE. The purpose of using a 75 g/L concentration of SCS was to see if a threshold in efficient Sg/YE ratio (the most efficient until now being a Sg/YE ratio of 20) had been surpassed with control A2 (Sg/YE ratio = 33). After several attempts targeting 75 g/L on WGP free sugar extraction, 75.7 g/L in SCS (Sg/YE ratio = 30.3) could be obtained through free carbohydrate water extraction of WGP and the solution was then added into the medium used for this experiment, named B1 from now on.

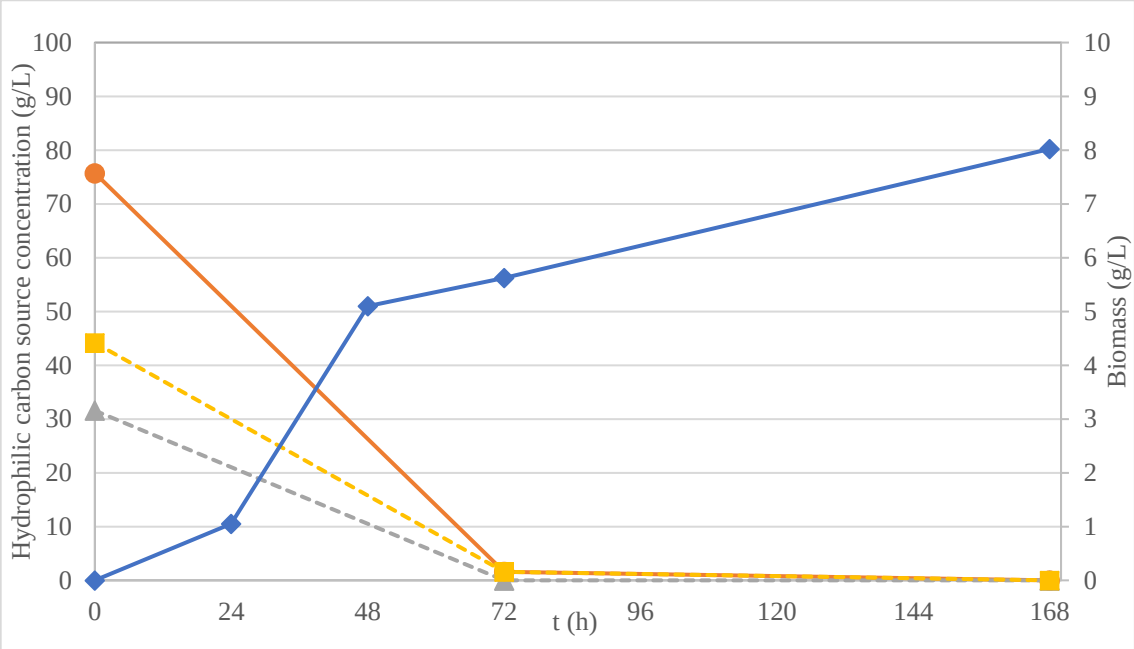

Figure 3.9 - Graph depicting experiment B1. Hydrophilic carbon source initial concentration is 75.7 g/L, read on primary Y axis and final biomass is 8.0 g/L after 168 h, read on the secondary Y axis. Squares – fructose; triangles – glucose; circles - glucose and fructose; diamonds – biomass.

Results of experiment B1, as observed in **Figure 3.9**, were also interesting. One can compare the results of this experiment with experiment A2 (**Figure 3.8**), in terms of the growth profile from 72 h to 168 h. Up to this point, behavior is exactly the same both in carbon source consumption as well as biomass production. From 72 h forward, however, the growth in biomass for B1 is much more accentuated than what was seen on A2. Biomass could be contaminated with oils, as no hexane wash step was performed on the pellets themselves as it was assumed that, upon intense centrifugation,

oils would be present as an organic phase on top of the aqueous supernatant or had been removed when washed with ethyl acetate. It might also be possible that this is related to the presence of microparticles of WGP that could still be present in the WGP SCS solution, which can be readily solved by efficiently filtering the solution before adding it to the culture media, instead of simply using multiple centrifugations to remove suspended particles, when retrieving the supernatant. It might also be that SL were still binding to the yeast cells' membranes, despite the wash with ethyl acetate when determining yeast dry weights. This factor should have even bigger influence on the last sample retrieved (168 h) as the corresponding volume is the entirety of the culture media, whereas on other daily samples, only a small volume is retrieved. Ultimately, the yeast cells could be storing something within their cytoplasm, increasing their overall weight.

Despite the biomass values being somewhat outlying, the SL production yields for experiment B1 were quite promising. At 168 h, SL production was at 35.0 g/L, the highest level of production obtained thus far (**Table 3.7**). Fed-batch reactor builds are known to obtain much higher yields, although at the cost of constant supplementation and more complex media (Davila et al., 1992). There are, however, authors that obtained similar yields using different and more commonly used hydrophobic substrates at 120 h of growth (Ul et al., 2018).

Table 3.7 - Table with SL production in g/L, and calculated yields in g/g monosaccharides and g/g biomass, for experiment B1, at 72 h and 168 h.

Time	g/L	g SL/g sugar	g SL/g biomass
72 h	10.2	0.14	1.81
168 h	35.0	0.46	4.36

After observing such an increase in SL yield, it seems that a Sg/YE ratio = 30 is beneficial to a larger scale production of SL. If this is to be true, this work might have come one step closer to optimal shake flask experiment conditions for WGP media, when looking to improve SL production. The oddity in this result is that no more glucose was present in B1's fermentation medium at 72 h and yet the SL production grew from 10.2 g/L to 35.0 g/L. As a last opinion for this disparity of results, it could be possible that SL recovered and weighed at 168 h were still contaminated with WGP oils in their midst despite the multiple washes with hexane and the absence of a yellowish color on the residue and the hexane wash supernatant already being clear at the time of weighing. In order to try and determine if this was a reality, oil conversion rates were calculated and are shown in chapter **3.4.1**.

This being said, the next step was to try and fortify this claim or refute it by improving SL yield further, increasing the amount of extracellular glucose available for the yeast cells to include in their SL synthesis pathway.

Experiment B2 was planned to this effect, with a strictly WGP medium of 97.6 g/L SCS, with all remaining components previously used at the same concentration. This would allow for a

Sg/YE ratio close to 40 that, in theory, should allow for a bigger margin in glucose for the yeast cells to use in producing SL.

The sugar consumption observed throughout the assay was somewhat unusual in that, despite the abundance at almost 100 g/L of initial SCS only 2.1 g/L of glucose and 3.8 g/L of fructose were available in the media, at 72 h. Although still a larger concentration than on experiment B1 (**Figure 3.9**) at the same point in time (glucose was fully consumed while fructose was 1.6 g/L), one would hope to see similar behavior to that of experiment A2 since at an initial concentration of 82.5 g/L there was still around 13.3 g/L of fructose and 6.1 g/L of glucose at 72 h. The results of experiment B2 were therefore somewhat unexpected because conditions were essentially the same for both B2 and A2: pH was kept steady at 3.5, temperature was 30 °C and stirring was kept at 180 rpm during the entire length of the assay. Also, no contamination could be detected in microscopical analysis of any of the cultures. The growth kinetics and sugar consumption curves for experiment B2 are displayed in **Figure 3.10**. Curiously, final biomass (7.8 g/L) is comparable to the biomass obtained in experiment B1 (8.0 g/L), since both growth medias were based of WGP extracts, had the same concentration of YE (2.5 g/L) and WGP particles could be present in both. Furthermore, SL production was also lower than experiment B1, only reaching 27.8 g/L at 168 h (**Table 3.8**) against B1's value of 35.0 g/L at the same point in time. Nevertheless, experiment B2 still had higher production than experiment A2, at 21.1 g/L.

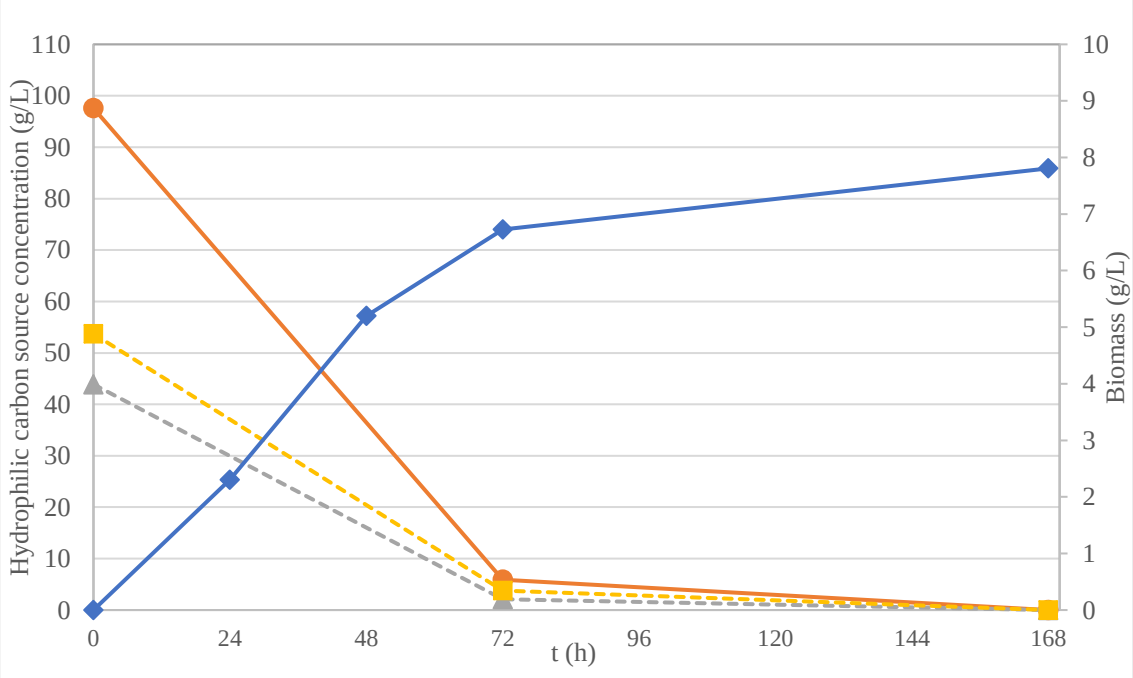

Figure 3.10 - Graph depicting experiment B2. Hydrophilic carbon source initial concentration is 97.6 g/L, read on primary Y axis and final biomass is 7.81 g/L after 168 h, read on the secondary Y axis. Squares – fructose; triangles – glucose; circles - glucose and fructose; diamonds – biomass.

Table 3.8 - Table with SL production in g/L and g/g monosaccharides and g/g biomass yields of SL production, for experiment B2, at 72 h and 168 h.

Time	g/L	g SL/g sugar	g SL/g biomass
72 h	8.2	0.11	1.22
168 h	27.8	0.29	3.57

To facilitate comparisons between experiments A and B, a summary table of all experiment yields in SL and monosaccharide concentrations at 72 h is shown in **Table 3.9**.

Table 3.9 - Summary table of initial SCS, glucose and fructose (72 h) and SL (168 h) concentrations in g/L.

	A1	A2	B1	B2
Initial SCS (g/L)	47.9	82.5	75.7	97.6
Glucose (72 h, g/L)	3.6	6.1	0	2.1
Fructose (72 h, g/L)	3.4	13.3	1.6	3.8
SL (168 h, g/L)	N/A	21.08	34.96	27.83

This small difference in terms of results between experiments B1 and B2 encouraged a search for the cause of the variation when SL yields are the concern. To try and answer this question, HPLC analysis were repeated, probing for other chemical species known to be produced by *S. bombicola*: mannitol, glycerol and ethanol, in fermentation media (Ferreira, 2018). Comparative production curves of fermentation by-products for B1 and B2 are shown in **Figure 3.11**.

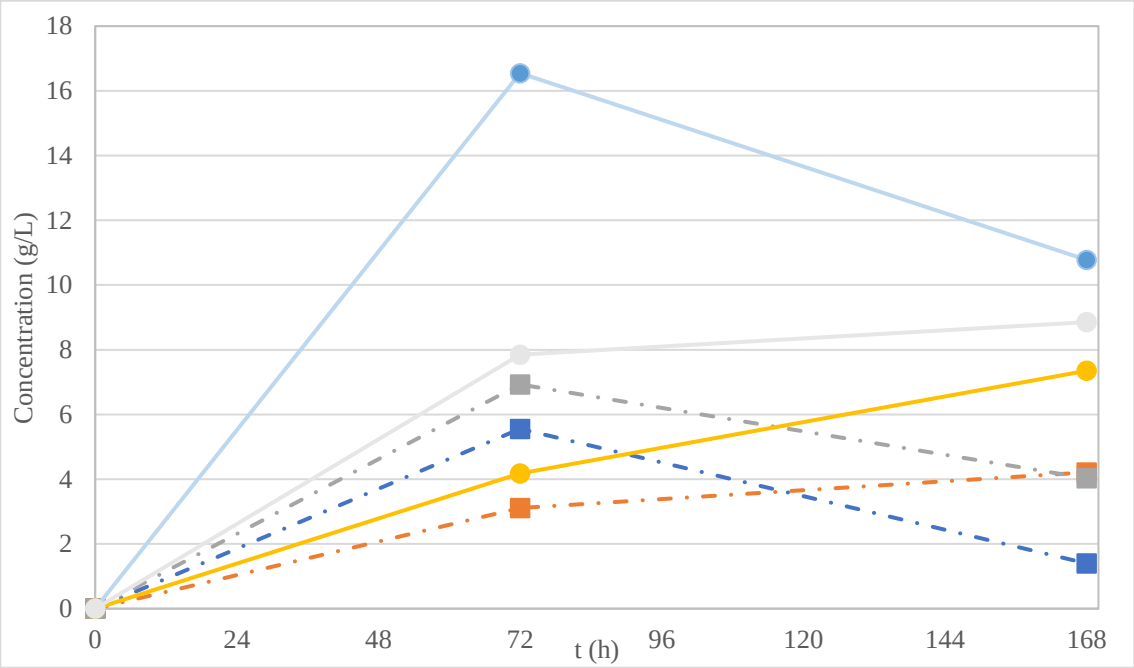

Figure 3.11 - Graph depicting fermentation by-products of experiments B1 and B2. Broken lines and square markers - B1. Full lines and circle markers - B2. Blue – ethanol, grey – glycerol, yellow/orange – mannitol.

Interestingly enough, one can see that experiment B2 (97.6 g/L initial SCS) had a larger by-product concentration across the board. Another interesting aspect is that, after 72 h, ethanol concentration on B2 fermentation media decreased significantly which should would go on par with the proposal that ethanol could have been consumed thus justifying the 168 h value of biomass for this experiment. Yet, if this were to be true, it would also mean that the 2.5 g/L of YE initially present in the fermentation medium had not been fully consumed by the 72 h mark, meaning nitrogen could still be present.

Turning the focus onto mannitol and glycerol, these are clearly still being produced after 72 h in experiment B2 whereas in experiment B1 all species except mannitol were already being consumed from 72 h to 168 h. This could be due to the availability of some glucose and fructose, granted at small concentration, at 72 h in B2's fermentation medium while it was expended completely in B1's fermentation medium, at the same point in time.

3.4 Sophorolipid purification and characterization

3.4.1 Fermentation product purification

The purification of obtained SL from the fermentation media was only done through washing steps with n-hexane with as many volumes as needed to obtain the oils still present in mixture with SL. These wash steps were also made in order to obtain a percentage of WGP OCS conversion, although this analysis was only done for experiments B1 and B2. Values of wt. % oil conversion are displayed in **Table 3.10**.

Table 3.10 – Initial OCS supplementation (g) and OCS recovered after hexane wash step (g) from the fermentation media of B1 and B2. OCS conversion rate in wt. % is also shown.

	Initial OCS (g)	Final OCS (g)	OCS conversion wt. %
Experiment B1	10.43	2.84	72.8
Experiment B2	9.56	1.52	84.1

From the oil conversion rates, one can say that the OCS supplementation was in excess to that needed for the obtained yields of SL for experiments B1 and B2. This might mean that if more glucose was available for the yeast cells to use when producing SL, yields could be further enhanced. Because of this it can be proposed that, for future experiments, an additional supplement or a higher initial concentration of WGP SCS should be used and that by doing so, it might lead to increased yields in SL while also facilitating the procedure of purification by allowing all the OCS that was still found in experiments B1 and B2 fermentation media to be fully consumed. Furthermore, these results do not prove that the conversion of oils was done solely towards the production of SL.

It also presents a possible validation to the previous hypothesis that the SL recovered from experiment B1 could still be contaminated with some amount of WGP oils because the culture period of both B1 and B2 was the same and yet the OCS conversion rate was relatively higher in experiment B2.

3.4.2 Structural characterization of the sophorolipids

3.4.2.1 LC-MS analysis

To verify that purified SL produced by *S. bombicola* in OCS supplemented media were successfully integrating the fatty acids present in WGP oil, an LC-MS analysis was performed. Focus was put on oleic acid and linoleic acid moieties (C18:1 and C18:2, respectively), even though stearic acid and palmitic acid moieties (C18:0 and C16:0 respectively) were also searched for but results are not shown as they require further analysis. To that end, samples were introduced in the binary pump

and negative ion spectra were obtained in the range of 0 m/z to 1000 m/z for the SL mixture. The SL mixture produced from OCS supplemented media appeared as a composition of both acidic SL (acC18:2, m/z = 703; acC18:1, m/z = 705 - **Figure 3.12**) and lactonic SL (lacC18:2, m/z = 685; lacC18:1, m/z = 687 - **Figure 3.13**).

Retention time of acC18:2 was 2.66 min and acC18:1 was 3.23 min while lacC18:2 had a retention time of 6.53 min and lacC18:1 of 9.18 min, as can be stated in the TIC (**Figure A 1**).

Despite their presence being confirmed, one might notice that lacC18:2 and lacC18:1 have relative absorbance intensities much lower than 100 %, around 15 % for lacC18:2 and 12.5 % for lacC18:1. The reason behind this was that the most intense peaks found were of m/z = 731 at 6.53 min and m/z = 732 at 9.18 min, respectively; these peaks can be perceived in **Figure A 2**.

These ions, however, couldn't be identified when integrating the mass spectrum signals and should be investigated in future work.

The most interesting aspect is that on earlier retention times, where characteristic signals of acidic SL are found (Hu and Ju, 2001), there appears to be no evidence of the presence of acidic conformers of the unidentified ions. However, by just the m/z ratio of these ions, one can only hypothesize that maybe a functional group or chemical species with relatively low molecular weight could've formed a bond to acidic SL molecules on one or more of their carboxylic extremities, after negative ionization by deprotonation like it is known to happen when executing the molecular ionization via ESI method (Banerjee and Mazumdar, 2012). One must also take into consideration that the ESI mode is known to have problems analyzing complex mixtures, sometimes offering unreliable results (Chemistry LibreTexts, 2019).

The MS device was also operated on positive ion mode, but these results are not shown.

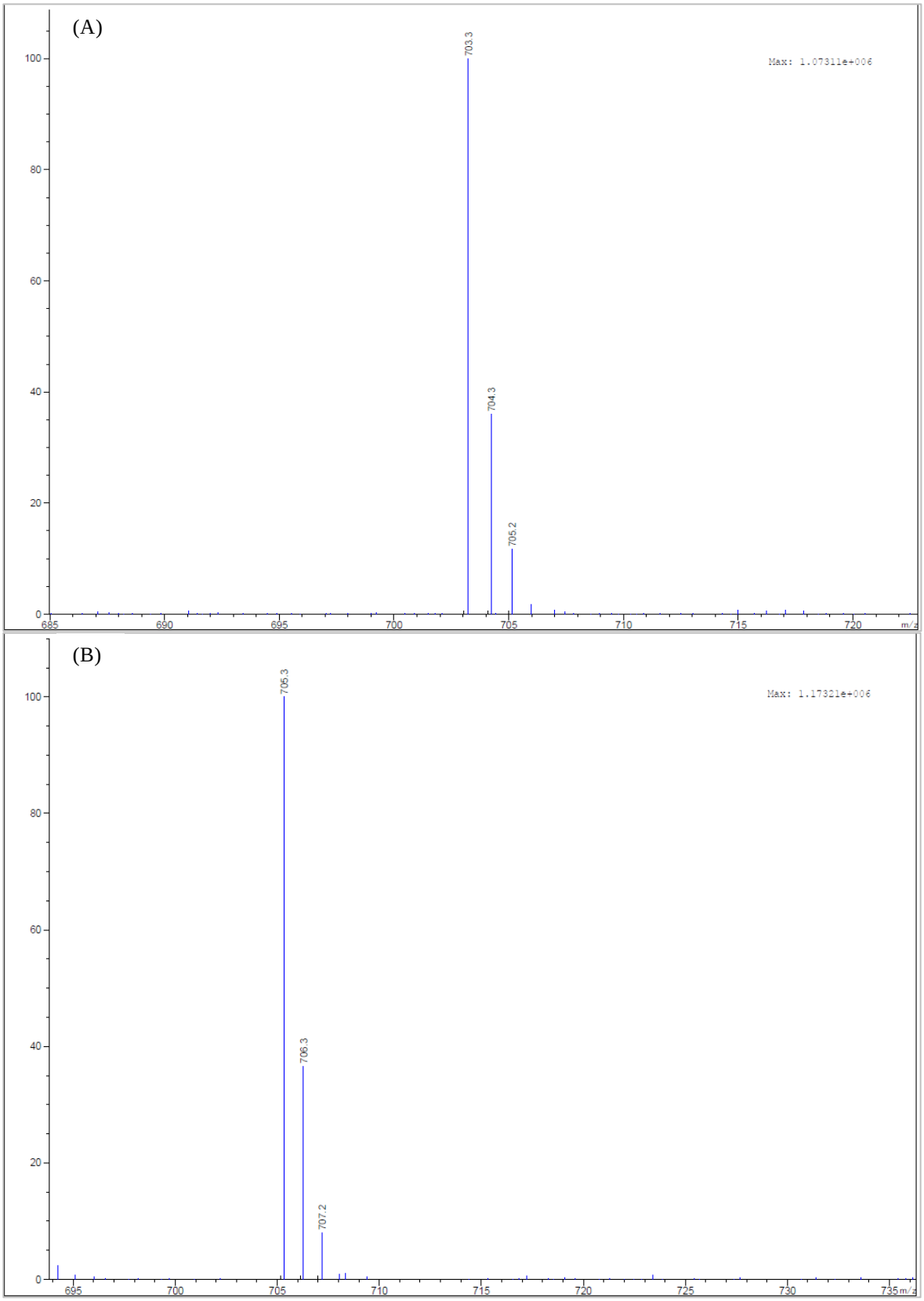

Figure 3.12 – Mass spectra of (A) acidic SL with linoleic acid moiety - acC18:2 and (B) acidic SL with oleic acid moiety - acC18:1, produced from OCS supplemented media.

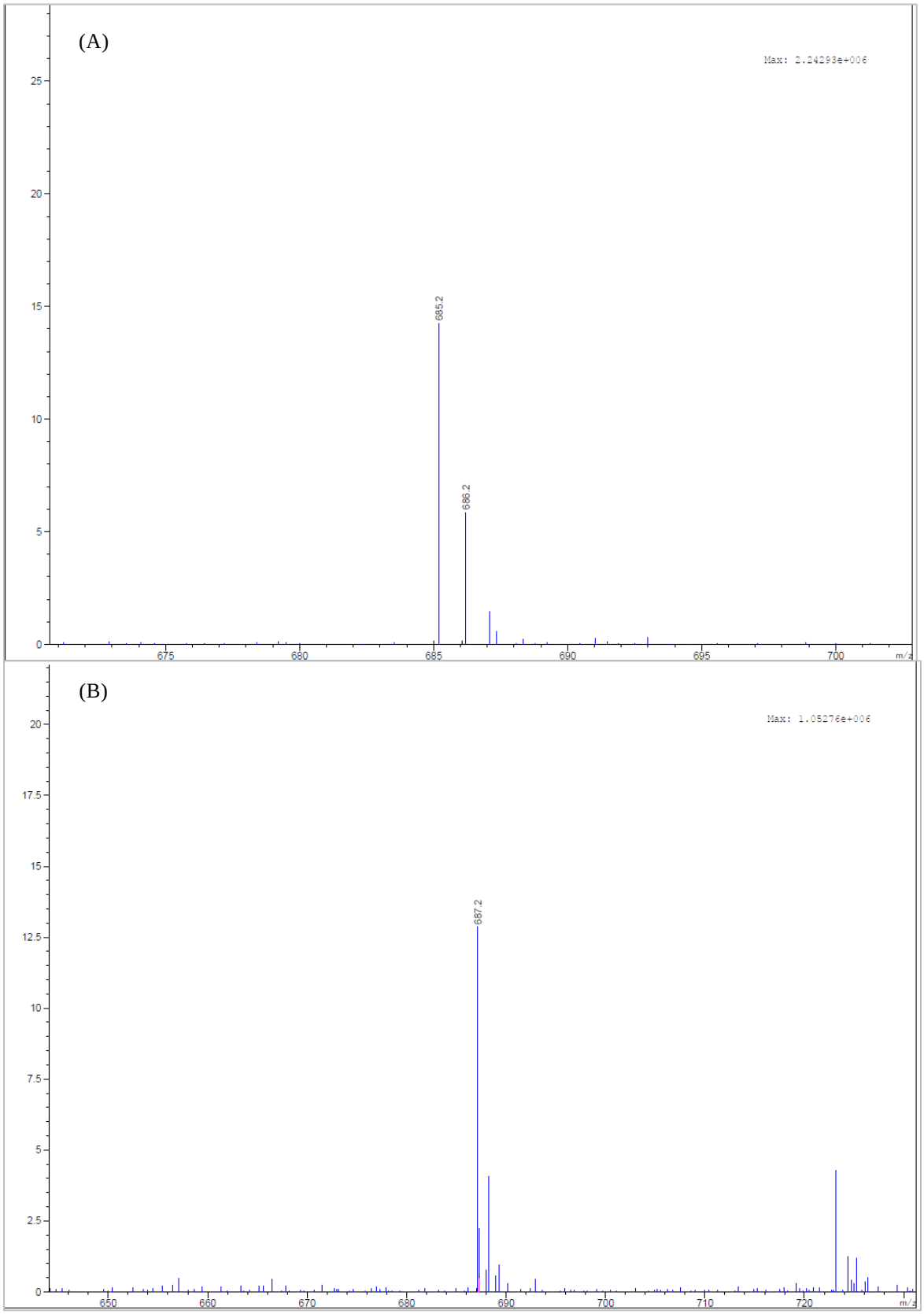

Figure 3.13 - Mass spectra of (A) lactonic SL with linoleic acid moiety -lacC18:2 and (B) lactonic SL oleic acid moiety - lacC18:1, produced from OCS supplemented media.

3.4.2.2 ^{1}H-NMR: SL structure estimation

The ^{1}H-NMR spectra of SL recovered from growth assays in fermentation media without OCS supplementation were assigned to typical glycolipid structures (**Figure A 3**). The large peak at approximately 1.3 ppm can be attributed to 18C of a fatty acid chain moiety typical of oleic (C18:1), linoleic (C18:2) and stearic acid (C18:0).

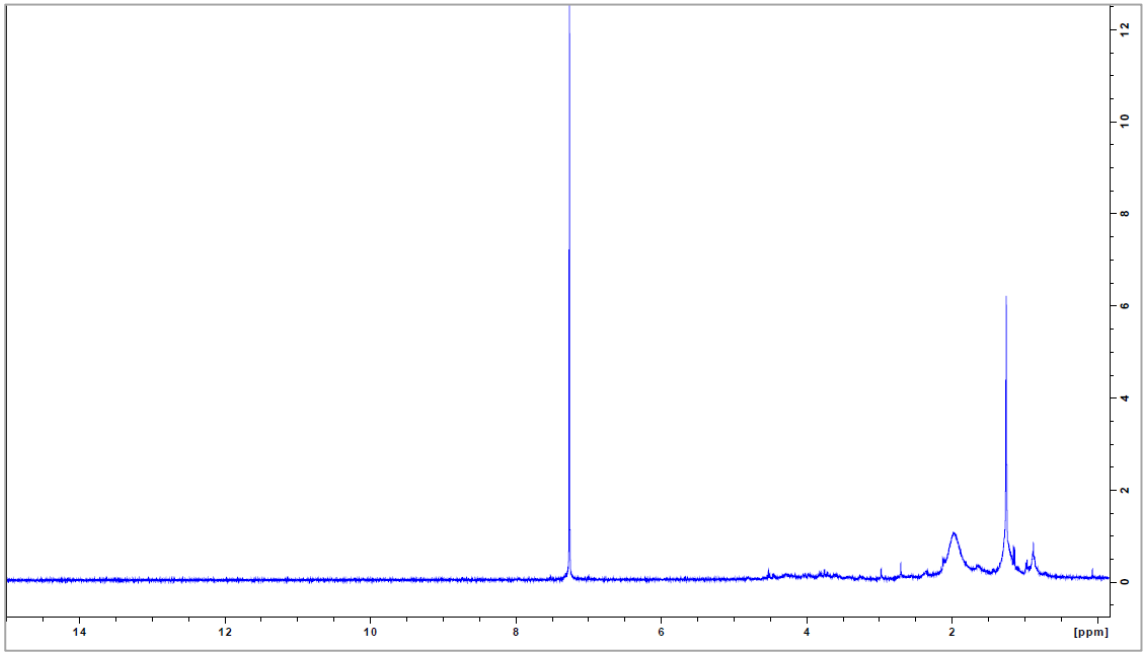

Figure 3.14 – ^{1}H-NMR spectrum of SL sample of experiments without WGP OCS supplementation (control A, control B and B0).

The length of the fatty acid chain gives rise to the signal at 1.2 ppm in the spectrum (**Figure 3.14**) as in agreement with literature, which states that *S. bombicola* has a preference for incorporating oleic acid moieties when "de novo" synthesis of SL occurs (Saerens et al., 2015). Moreover, the slightly round peak found at 2.0 ppm, probably corresponding to a combination of several peaks with a small ppm difference that the resolution of the used method did not allow to distinguish, is usually attributed to carbons 2-16 of the fatty acid hydrocarbon chain as well as 6' and 6'' acetyl (-$COCH_3$) modification of the sophorose, tipically found in lactonic SL as a distinct peak at 2.09 ppm. The protons of both glucoses that comprise sophorose show signals integrated around the 3.5-4.5 ppm area; these peaks can be more precisely spotted on the top panel of **Figure A 3**.

After observing the presence of SL-typical proton resonances in samples originating from SL production on only hydrophilic carbon sources the followthrough procedure was to investigate if differences could be spotted on the ^{1}H-NMR spectrum of SL mixture samples obtained from WGP OCS supplemented media; this spectrum can be seen on **Figure 3.15**.

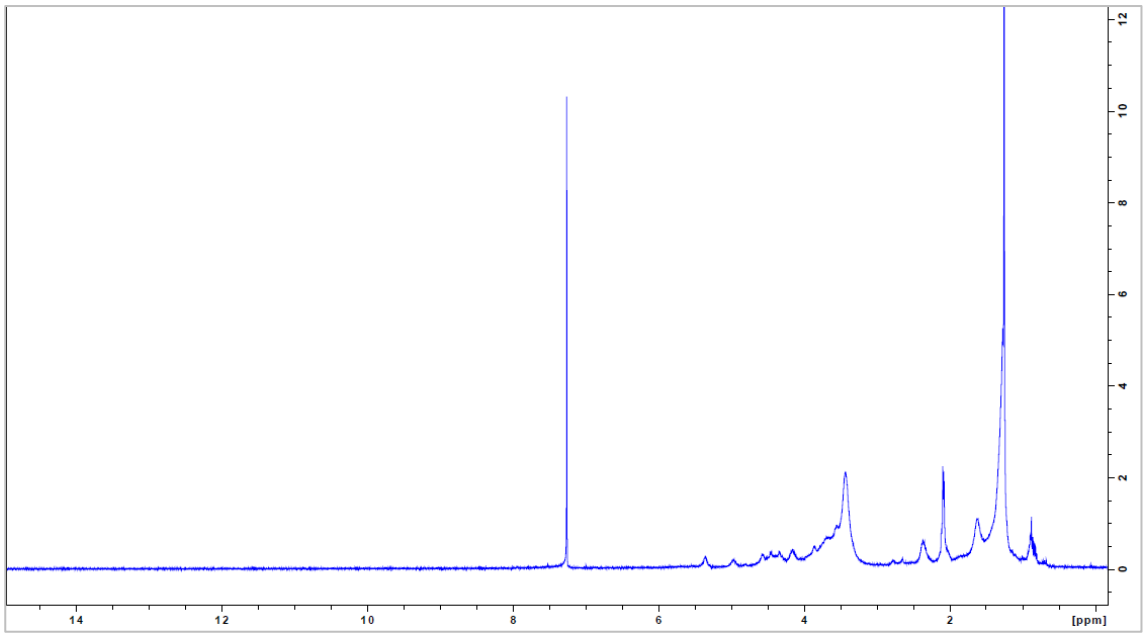

Figure 3.15 -^{1}H-NMR spectrum of SL sample of experiments with WGP OCS supplementation (A1, A2, B1 and B2).

The sample representing all SL obtained from fermentation media supplemented with WGP OCS shows a clearer resolution due to the peaks having higher intensities. The explanation for this is that this particular sample is surely richer in a variety SL than the one obtained from media without OCS supplementation and so signals show higher intensity as there are more proton interactions being detected by the NMR probe.

Figure 3.15 clearly shows pronounced signals around 1.2 ppm, as previously seen on the results of the WGP OCS-free sample once more confirming the presence of, presumably, an eighteen carbon long fatty acid chain since the most common fatty acids present in the profile of WGP OCS are C18:2 and C18:1. It might be possible that C16:0 and C18:0 were also incorporated (Saerens et al., 2015) due to their relative availability in WGP oils.

Again, several peaks around 2 ppm can be assigned to the several carbons of the fatty acid hydrocarbon chain. The large wide peak around 3.5 ppm seems to be overlapping smaller peaks, possibly all associated to the presence of glucose di-saccharide, sophorose.

3.4.3 Surface tension: critical micelle concentration and minimal surface tension

The final tests to characterize SL produced in media with OCS supplementation were surface tension essays, in order to obtain critical micelle concentration (CMC) and minimum surface tension (MST) values. **Figure 3.16** represents CMC and MST values of MilliQ solutions containing SL at gradient concentrations.

The SL retrieved from WGP fermentation media presented values that were in accordance with literature for this type of biosurfactant, when produced in unorthodox media (Daverey and Pakshirajan, 2009). The same authors later obtained a better CMC value of 27.17 mN/m when using a mix of carbon sources, in other experiments using glucose as hydrophilic carbon source and pure oleic acid as hydrophobic carbon source (Daverey and Pakshirajan, 2010).

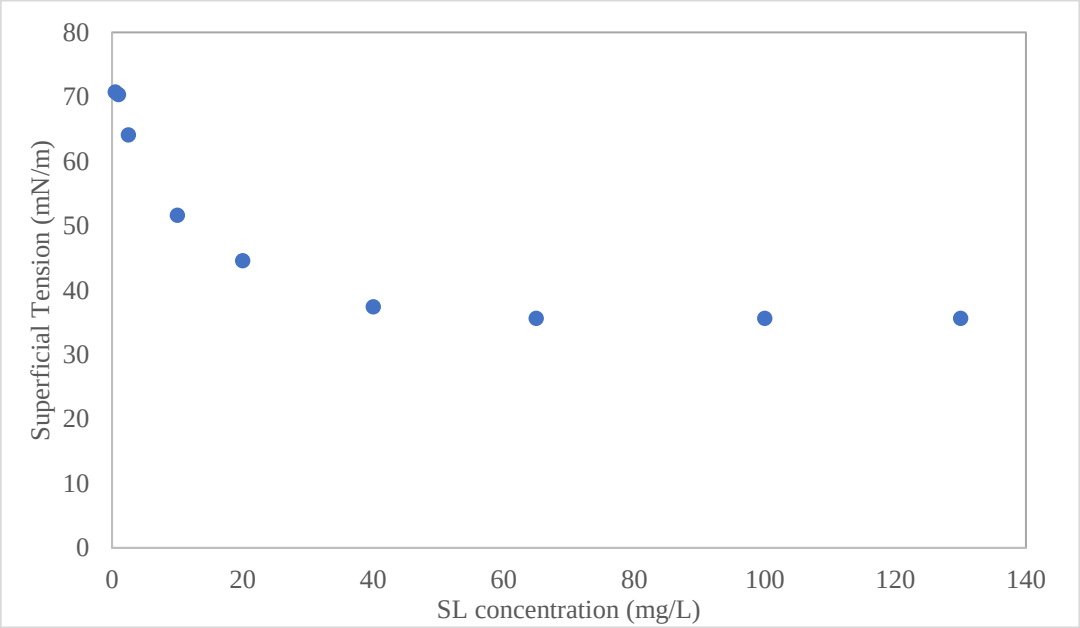

Figure 3.16 – CMC and minimum surface tension of different SL concentrations in a MilliQ solution produced on OCS supplementation experiments.

In this experiment, a CMC value of 53.79 mg/L was obtained from a graph of surface tension against log SL concentration (**Table A 2**), slightly higher than the above referenced literature. Yet the MST value obtained for these SL was 35.62 mN/m. Reviewing these results, one can say that in spite of needing a higher concentration to attain the most surface tension reduction, the SL recovered from experiments using WGP OCS are considered good biosurfactants, almost as good as the SL obtained by Daverey and Pakshirajan (2010) whom obtained a MST value of 34.18 mN/m, thus making them suitable candidates to be used in future biodegradable detergents, despite needing somewhat higher concentration to achieve the same performance in terms of tension reductive properties, on par with more conventional detergents like dodecyltrimethylammonium bromide, MST = 51 mN/m (Kulakovskaya and Kulakovskaya, 2014).

4 Conclusion

The aim of this work was to repurpose white grape pomace (WGP), an agro-industrial waste that exists in abundance known to possess high levels of free sugars and some lipids originating from their presence in seeds, pulp and stalks that are not incorporated in the fermentation of white wine. The proposed target of repurposing was to use WGP extracts as a microbial substrate for *S. bombicola*, a type of yeast known to be successful in producing value-added products known as sophorolipids (SL).

The entire method was initially planned to be as sustainable and green as it possibly could and so, to ensure this, supercritical carbon dioxide extraction ($scCO_2$ extraction) was proposed as the chosen technique of oil extraction whereas a simple water extraction at moderate temperatures (35 °C-40 °C) was chosen to extract the free sugars present in oil-free WGP.

A minor characterization of WGP was performed which allowed to conclude that indeed lipids were present in a range of around 10 to 14 wt. %, mostly depending on the amount of seeds present of the sample, and that their profile in fatty acids, acquired through GC-FID, was within boundaries already set in literature as linoleic acid (≈ 69 %) was the most present of the four fatty acids found, the others being oleic acid (≈ 15 %), palmitic acid (≈ 11 %) and stearic acid (≈ 8 %).

Through HPLC analysis, the contents in free sugar of WGP were found to be high, around 43 %, with glucose and fructose comprising the highest percentage of free sugar at around 21 % and 22 % respectively, as expected since these two monosaccharides are known to be found in the same quantity in WGP. The maximum concentration of free sugar obtained upon direct extraction from WGP was 75.7 g/L (glucose + fructose). However, combining the water extraction with lyophilization allowed working with a solution having 97.6 g/L of glucose + fructose.

The $scCO_2$ extraction was able to retrieve oils from WGP although not their entirety; the maximum extraction yield was around 4.4 % out of a maximum of 14 % of oil, obtained through Soxhlet extraction and oil dry weight measurement. This might have occurred due to the size of the particles inside the reactor being too large, which prevented proper CO_2 diffusion within the solids or because the temperatures of 40 °C and 45 °C that were used were not ideal to fully recover the oils. Because of the lack of efficiency of the extractions performed in this work and because the purpose of this work was to exhaust as many bioactive compounds still present in WGP as possible, the method chosen for extracting oils to use in yeast cultures, or oil carbon sources (OCS), was the Soxhlet method.

When growing *S. bombicola* PYCC 5882 on WGP SCS, the attention went into an efficient medium production. To select the most appropriate media, the sugar to yeast extract ratio (Sg/YE) was considered whenever choosing new conditions for a yeast growth assay. The yeast behaved as expected, consuming the free sugars present in the media naturally and in agreement with the fructophilic aspect of this genus of yeast. As expected on only hydrophilic carbon sources, production

in SL was not high, having reached a maximum of 3.4 g/L at 72 h on a medium with a Sg/YE ratio of 20.

Experiments using media combining both free sugar and oil from WGP, at different SCS concentrations and 50 g/L OCS, were successful in obtaining greater yields in SL than when *S. bombicola* was grown solely on hydrophilic carbon sources. The yeast cells thrived in the presence of WGP oils, producing a maximum of 35.0 g/L on a medium with a Sg/YE ratio of 30, in experiment B1. Experiments A2 and B2, with Sg/YE ratios above 30, did not manage to achieve the same yield in SL. Concluding, WGP oil extracts are suitable to be used as supplementary hydrophobic carbon source when attempting to produce higher yields in SL.

Further HPLC analysis was done to figure out why a higher concentration of free sugars while maintaining both oil and yeast extract concentrations the same led to a lower yield in SL. Mannitol, glycerol and ethanol are typical by-products of *S. bombicola* metabolic pathways and most of all they require the presence of glucose within the cell to be produced. It was conclusive that a part of the glucose supplied in the media was being directed to these metabolic pathways, especially on experiment B2, with the highest initial content in glucose and fructose (97.6 g/L). Ethanol, glycerol and mannitol were all produced to a larger extent in B2 than B1, ethanol particularly being present in concentrations almost three folds higher. This fact could very well be one of the reasons why B2 consequently produced less SL than its counterpart experiment, B1.

What one can state in regard to the fermentation assays performed is that the Sg/YE ratio of 30 of experiment B1, at 75.7 g/L of WGP sugar carbon sources (SCS) appeared to deliver the best SL yield (35.0 g/L) from the combination of experiments performed. *S. bombicola* appeared to prioritize the synthesis of SL over the other mentioned by-products, found at a lower concentration at 168 h when compared. Nevertheless, there is surely still much room for improvement and further assays should be performed to test optimal growth conditions with WGP extracts.

Sophorolipid purification seems to not have been completely successful. By just adding hexane wash steps after recovering from the fermentation media, visually, no oil seemed to be present after several washes with hexane, but some results might make the reader doubt the purity of the SL obtained. Since no SL standards were used in analytical approaches throughout the entire work, one cannot be assured that the SL mixture had no contaminations.

The structural characterization of the SL mixture was done via LC-MS and ^{1}H-NMR. According to the results obtained and understanding how *S. bombicola* behaves, it appears that both acidic (acSL) and lactonic sophorolipids (lacSL) were present and did indeed incorporate fatty acid moieties present in the WGP oil fatty acid profile. Although acSL are typically intracellular, they tend to be transported to the extracellular area to only then be converted into lacSL by *S. bombicola*'s lactone esterase. Combination of both ^{1}H-NMR and LC-MS results allowed to conclude that sophorolipids were present in the samples retrieved from fermentation media although mixed with other unidentified components.

The LC-MS analysis also revealed signals at m/z = 731 and m/z= 732 with relative intensities towering over lacSL signals found in the same retention times (6.52 min and 9.18 min respectively) when no evidence was found on literature of such ions existing. If such ions do indeed exist and are not the result of contamination will be left for future investigation to uncover. Also, palmitic acid and stearic acid are also present in WGP and can be incorporated in sophorolipid structures, as proven by various authors and should also be identified, if present in MS spectra, in future reports.

The final aspect of study on the produced SL was their ability to be used as detergents and micelle forming agents. For that, surface tension assays were performed on samples obtained from OCS supplemented media and the values for critical micelle concentration (CMC) and minimum surface tension (MST) were obtained. The SL produced had a CMC of 53.79 mg/L and a MST of 35.62 mN/m, putting them on par with other authors' obtained biosurfactants.

5 Future work

In future work regarding extraction of bioactive compounds from WGP, it should be paramount to optimize $scCO_2$ extraction performances in order to create a more sustainable method of oil extraction, preferably one that offers the same yields with non-pollutant solvents and hopefully at a quicker rate than standard Soxhlet extraction. For that, we propose as future work to increase extraction temperatures to 60 °C to fluidify the oils that could be retained inside the extractor and attempt different CO_2 flow rates to allow for better solvent diffusion. Adding to that, proper grinding of WGP solid should also help improve yields.

As for the water extraction of WGP free sugars, an efficient, quick and simple method was used in this work. However, to obtain higher concentrations of sugar, the high WGP mass used proves troublesome for retrieving the extracts, even when using high speed centrifugation for long periods of time. A workaround was proposed in this work, using vacuum lyophilization to obtain the sugars from solutions of lower concentration and then adding them up to a previous solution of known concentration but this method requires the use of a vacuum freeze-dry lyophilizer, a machine that requires a large amount of power to work and a process that takes some time. In the future, a solution to make the free sugar extraction more efficient and less time consuming should be tested.

Although characterization of SL in this work was as extensive as some authors, others use additional analytical approaches to characterize these biomolecules. One that is common practice amongst authors that focus on sophorolipid production and characterization is FT-IR. That, together with more effective purification methods should deliver more promising results in the future.

Regarding the yeast growth cultures, duplicates must be done in the near future in order to replicate results and to allow for more extensive discussion for a variety of growth conditions. Additionally, growth experiments with fatty acids present in the WGP oil profile individually should be performed and their incorporation in SL tested so as to compare results with individual species found on analysis of the SL mixture. It would also be interesting to use genetically modified strains of *S. bombicola*, optimized for SL production by perhaps deleting genes that are essential to metabolic pathways that diverge from the one that leads to SL production such as the pathway of glycerol production.

Possible applications for the retrieved SL should be tested. They are known to have several applications and their broad spectrum of utilities would be an interesting aspect to explore and test in future work.

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7 Appendix

Table A 1 – NHCS elemental analysis results for WGP free-sugar solution.

Element	Average %
Nitrogen	0.27 ± 0.02
Carbon	36.86 ± 0.04
Hydrogen	6.45 ± 0.12
Sulphur	0.00 ± 0.00

Table A 2 – Table of data used for CMC and MST calculations for different SL concentrations. Values are displayed alongside standard deviations of Huh-Mason method, obtained automatically with the tensiometer.

SL concentration (mg/L)	log [SL] (mg/L)	S. tension (mN/m)	Std. Dev.
0,5	-0,301029996	70,76	0,01
1	0	70,39	0,09
2,5	0,397940009	64,11	0,07
10	1	51,63	0,29
20	1,301029996	44,58	0,22
40	1,602059991	37,44	0,12
65	1,812913357	35,62	0,07
100	2	35,63	0,01
130	2,113943352	35,62	0,01

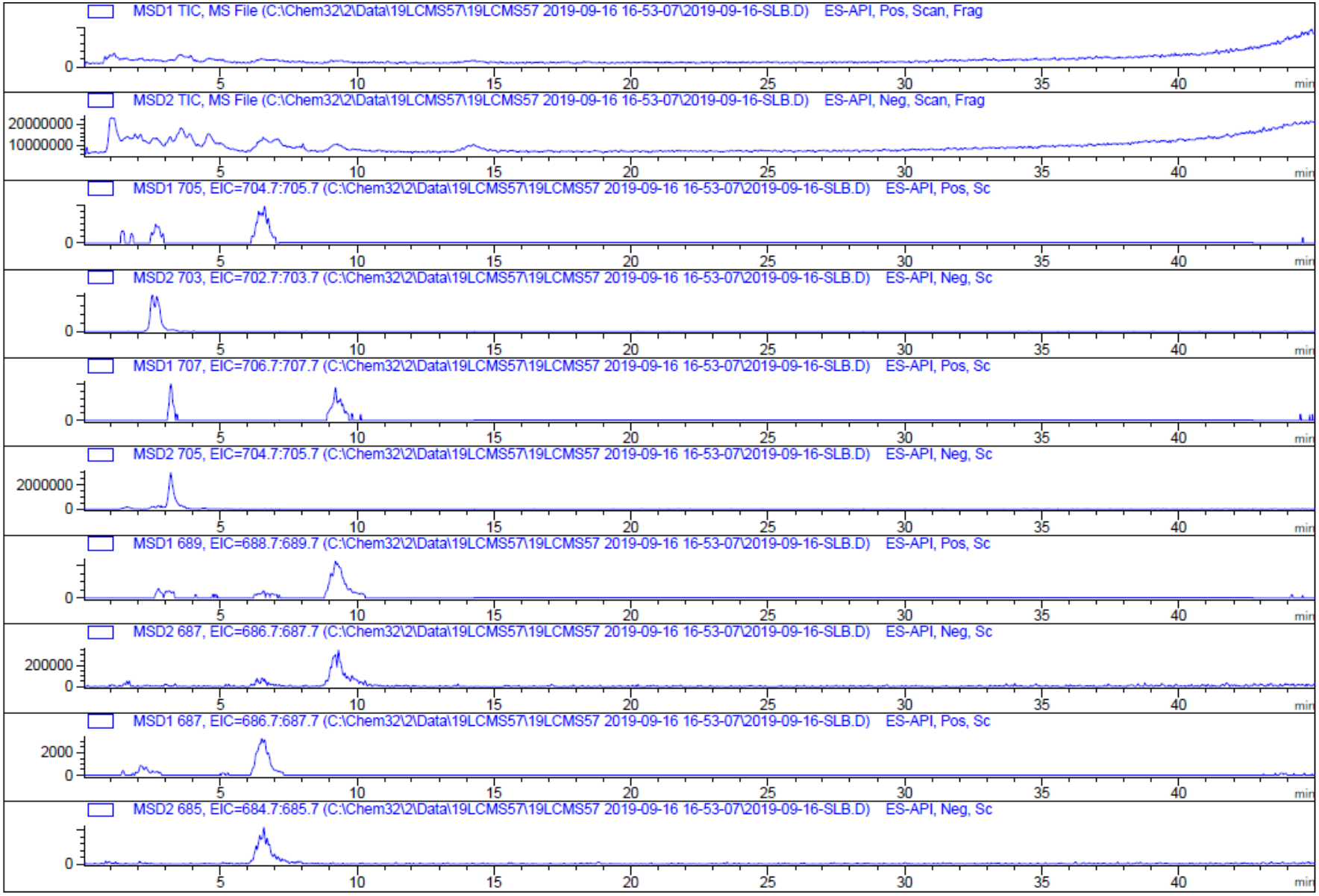

Figure A 1 – TIC of all species detected in MS-ESI assay. Both positive and negative ion modes are displayed.

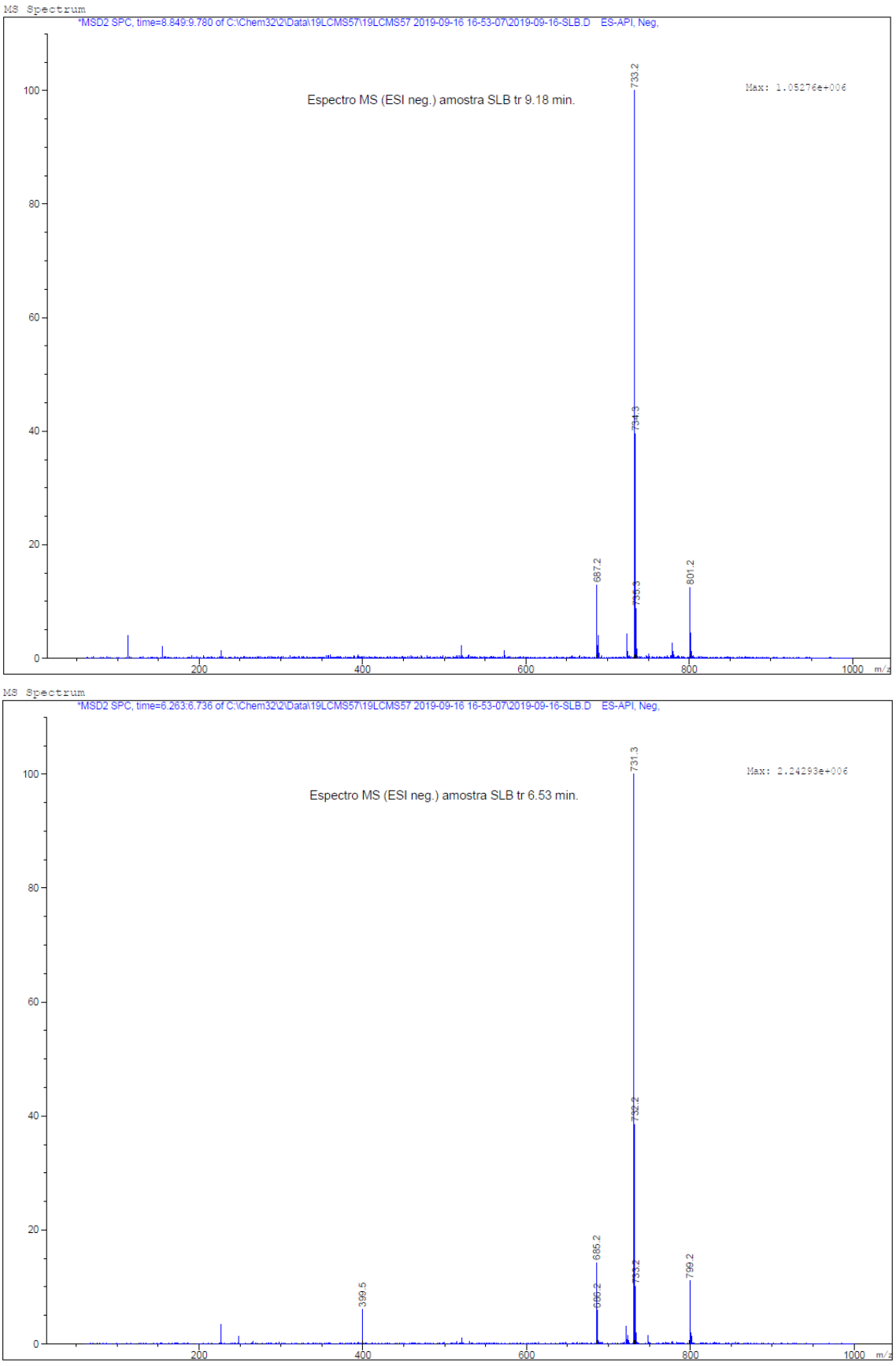

Figure A 2 - MS-ESI full m/z spectra of retention times 9.18 min (top) and 6.53 min (bottom). One can see the main negative ions aquired at both retention times, m/z = 733 at 9.18 min and m/z = 731 at 6.53 min.

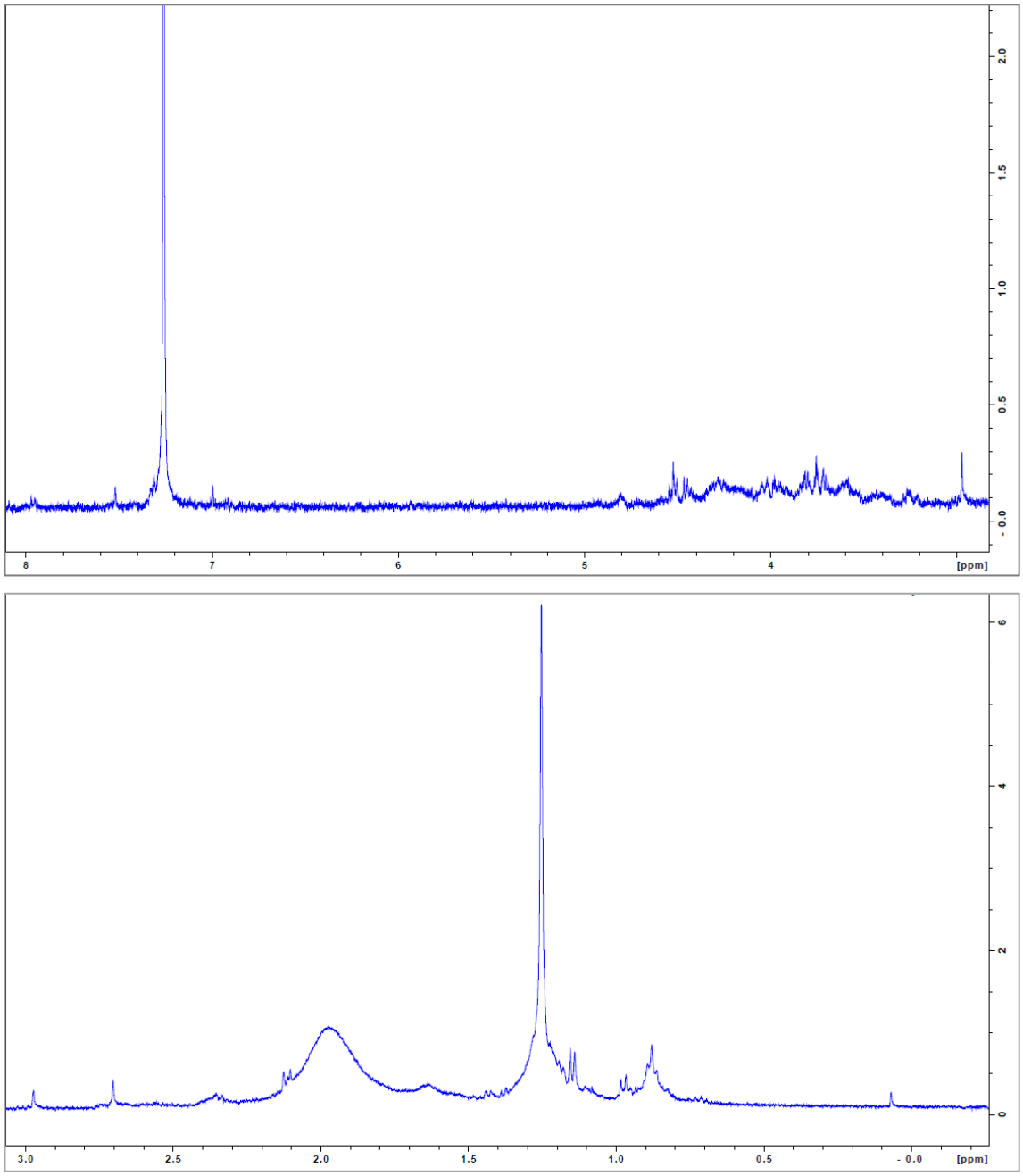

Figure A 3 – ^{1}H-NMR spectra, divided in two parts, of sophorolipids recovered from fermentation media with no OCS supplementation (control A, control B and B0). Top: 8-3 ppm. Bottom: 3-0 ppm.

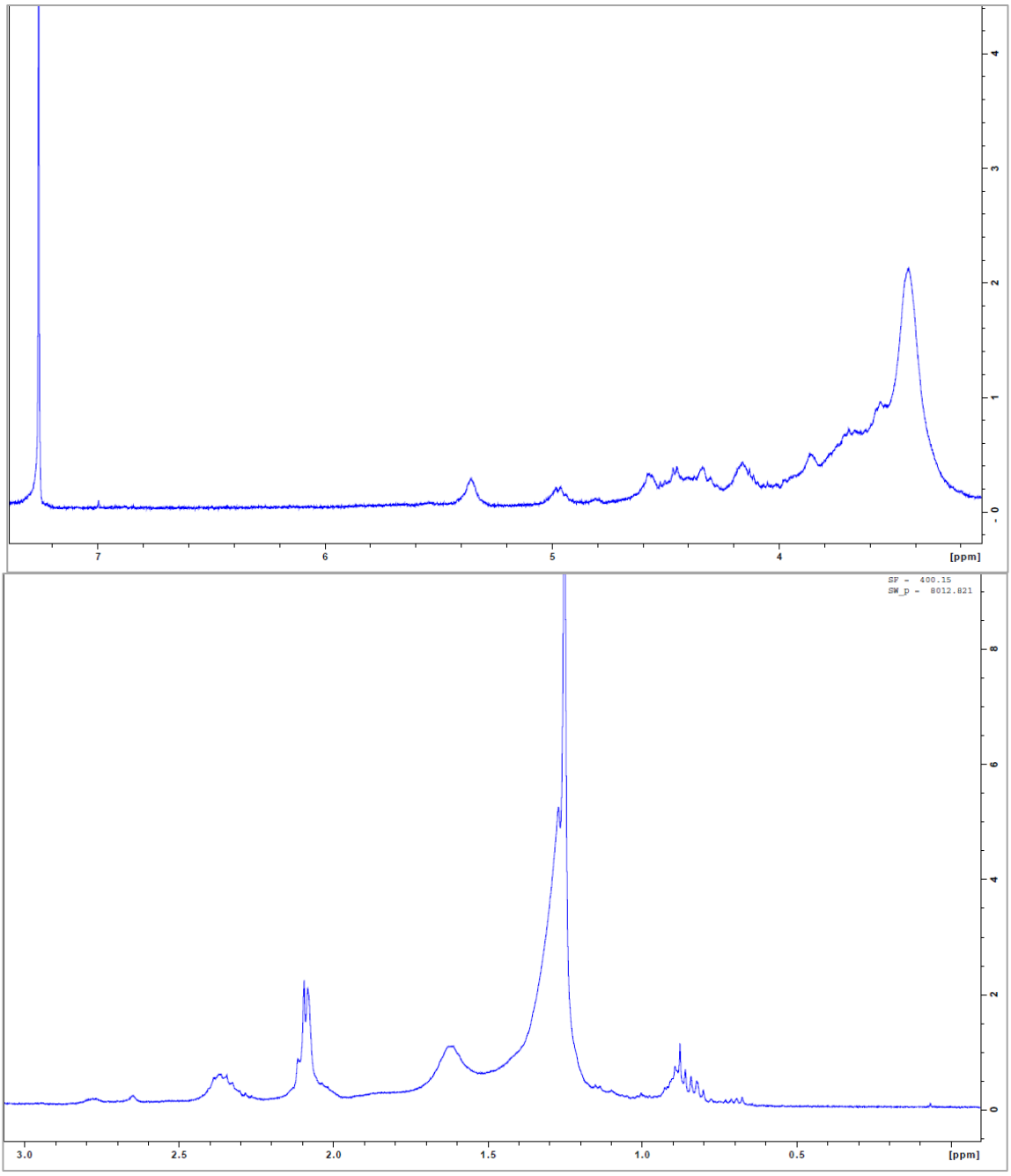

Figure A 4 – ^{1}H-NMR spectra, divided in two parts, of sophorolipids recovered from fermentation media with OCS supplementation (A1, A2, B1 and B2). Top: 8-3 ppm. Bottom: 3-0 ppm.

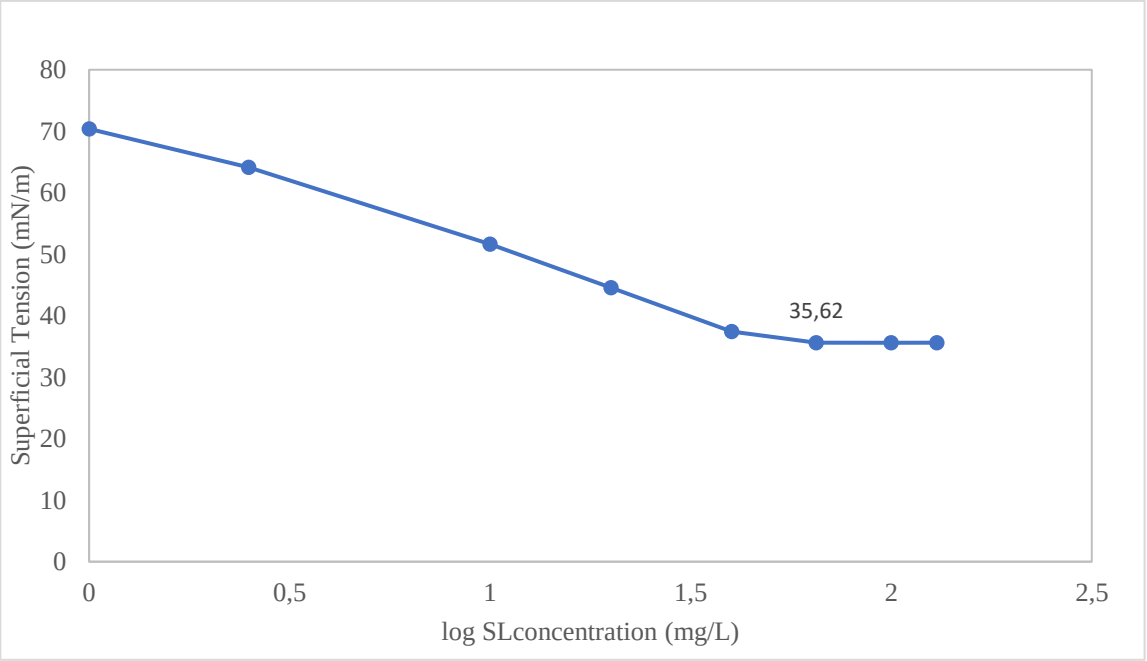

Figure A 5 - Graph of surface tension (mN/m) against log SL concentration. Used to retrieve CMC value.