NATALIA DE ANDRADE TEIXEIRA FERNANDES

Gene expression of the yeast *Wickernhamomyes anomalus* CCMA 0358 during biosurfactant production using kitchen waste oil as substrate and identification of bacteria present in tea tissue culture (*Camellia sinensis*) through metagenomics analysis

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Orientador

Prof. Dr. Disney Ribeiro Dias

Coorientadora

Prof. Dr. Jacquelyn Gervay-Hague

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APROVADA em 21 de maio de 2021.

Dr. Danilo de Menezes Daloso UFC

Dr. Jacquelyn Gervay-Hague UCD

Dr. Joyce Doria Rodrigues UFLA

Dr. Rosane Freitas Schawn UFLA

Prof. Dr. Disney Ribeiro Dias

Orientador

Prof. Dr. Jacquelyn Gervay-Hague

Coorientadora

LAVRAS – MG

2021

The memories of Zizinho and Zizinha, the best grandparents I could have, that encouraged my formal education and my personal growth.

To my parents for all the encouragement and understanding in the absence hours.

I dedicate.

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"Don't ever let someone tell you that you can't do something. You got a dream, you got to protect it. When people cannot do something themselves, they are going to tell you that you cannot do it. You want something, go get it." (The Pursuit of Happiness - Will Smith)

RESUMO

Os biossurfactantes são metabólitos secundários, de natureza anfifílica, com potencial para aplicação em detergentes, fármacos, cosméticos e na indústria alimentícia. O objetivo deste estudo foi purificar e identificar a estrutura do biossurfactante produzido pela levedura *Wickerhamomyces anomalus* (CCMA 0358) utilizando óleo residual de cozinha (KWO) como fonte de carbono e suas frações isoladas, bem como avaliar as alterações na expressão gênica durante a produção de o biossurfactante. O biossurfactante foi produzido em biorreator, utilizando meio de cultura previamente otimizado, com outras fontes de carbono, por 12h. O biossurfactante bruto e suas frações isoladas e purificadas (F1 e F2) foram caracterizadas por Cromatografia em Camada Delgada (CCD), Cromatografia Liquida acoplada a um Espectro de Massas (CL-EM) e por Ressonância Magnética Nuclear (RMN). Esperava-se encontrar um biossurfactante glicolipídeo, entretanto, nossos espectros mostraram que este biossurfactante não possui uma cadeia de açúcar em sua estrutura, sugerindo que se trata de um triglicerídeo. A análise de expressão gênica baseada em RNA-seq identificou mais de 6.000 genes expressos, nos quais 829 são expressos de forma diferente entre as amostras de controle (sem KWO) e tratamento (com KWO). As enzimas precursoras para a produção de biossurfactantes foram apresentadas neste estudo, tendo maior expressão em precursores das vias de metabolismo de lipídeos e ácidos graxos, onde uma das frações dos biossurfactantes é produzida. O biossurfactante bruto, F1 e F2 também foram avaliados contra a bactéria Gram-positiva *Bacillus cereus* e apresentaram boa atividade antibacteriana com MIC de 120, 240 e 60 µg/mL respectivamente e MBC de 480, 240 e 60 µg/mL, respectivamente. O chá (*Camellia sinensis*) é uma das bebidas mais importantes do mundo e fornece vários metabólitos secundários que são responsáveis por seu sabor rico e benefícios para a saúde. Em culturas *in vitro*, podem ocorrer inúmeras perdas por contaminação bacteriana, mas vários estudos indicam que os endófitos têm um efeito positivo nas plantas. Portanto, este estudo mostra a população de bactérias encontradas em duas variedades de cultura de tecidos de chá, utilizando a técnica metagenômica. No total, 17 DNAs bacterianos foram identificados, 10 dessas espécies foram identificadas em ambas as variedades, incluindo *Klebsiella pneumoniae*, que pode fixar o nitrogênio atmosférico em uma forma que pode ser usada pelas plantas, *Cronobacter sakazakii* frequentemente encontrado associado a plantas e rizosfera e está relacionado à solubilização de fosfato mineral e produção de ácido indol acético e *Paenibacillus* spp. onde muitos provaram ser importantes para aplicações agrícolas e hortícolas, industriais e médicas. Este estudo é o primeiro a correlacionar microrganismos associados ao cultivo *in vitro* de *C. sinensis*.

Palavras-chave: Expressão genica, RNA-seq, levedura, antibacteriano, cultura de tecidos, metagenômica, metabolitos.

ABSTRACT

Biosurfactants are secondary metabolites, of an amphiphilic nature, with potential for application in detergents, drugs, cosmetics and in the food industry. The aim of this study was to purify and identify the structure of the biosurfactant produced by the yeast *Wickerhamomyces anomalus* (CCMA 0358) using kitchen waste oil (KWO) as a carbon source and its isolated fractions, as well as to evaluate changes in gene expression during the production of the biosurfactant. The biosurfactant was produced in a bioreactor, using previously optimized culture medium, with a different carbon source, for 12h. The crude biosurfactant and its isolated and purified fractions (F1 and F2) were characterized by Thin Layer Chromatography (TLC), Liquid Chromatography coupled to a Mass Spectrum (LC-MS) and Nuclear Resonance Magnetic (NMR). It was expected to find a glycolipid biosurfactant, however, our spectrum shows that this biosurfactant does not have a sugar chain in its structure, but our spectrum suggests that it is a triglyceride. The analysis of gene expression based on RNA-seq identified more than 6,000 expressed genes, in which 829 are expressed differently between control samples (without KWO) and treatment (with KWO). The precursor enzymes to produce biosurfactants were presented in this study, having greater expression in precursors of the lipid and fatty acid metabolism pathways, where one of the fractions of the biosurfactants is produced. The crude biosurfactant, F1 and F2 were also evaluated against the Gram-positive bacterium *Bacillus cereus* and showed good antibacterial activity with MIC of 120, 240 and 60 µg/mL respectively and MBC of 480, 240 and 60 µg/mL, respectively. Tea (*Camellia sinensis*) is one of the most important drinks in the world and provides several secondary metabolites that are responsible for its rich taste and health benefits. In in vitro cultures, numerous losses due to bacterial contamination can occur, but several studies indicate that endophytes have a positive effect on plants. Therefore, this study shows the population of bacteria found in two varieties of tea tissue culture, using the metagenomic technique. In total, 17 bacterial DNAs have been identified, 10 of these species have been identified in both varieties, including *Klebsiella pneumoniae*, which can fix atmospheric nitrogen in a form that can be used by plants, *Cronobacter sakazakii* often found associated with plants and rhizosphere and is related to the solubilization of mineral phosphate and production of indole acetic acid and *Paenibacillus* spp. where many have proven to be important for agricultural and horticultural, industrial and medical applications. This study is the first to correlate microorganisms associated with the *in vitro* cultivation of *C. sinensis*.

Keywords: Gene expression, RNA-seq, yeast, antibacterial, tissue culture, metagenomics, metabolites.

SUMÁRIO

CHAPTER I

SECTION 1

ABSTRACT

Biosurfactants are secondary metabolites, of an amphiphilic nature with potential for application in detergents, pharmaceuticals, cosmetics and in the food industry. The aim of this study is to purify and identify the structure of the biosurfactant produced by the yeast *Wickerhamomyces anomalus* (CCMA 0358) using kitchen waste oil (KWO) as carbon source, as well as to evaluate the changes in gene expression during production of the biosurfactant. The biosurfactant was produced in a bioreactor, using a previously optimized culture medium, with a different carbon source, for 12h. The biosurfactant was recovered from the supernatant by adsorption chromatography. The crude biosurfactant and its isolated and purified fractions (F1 and F2) were characterized by Thin Layer Chromatography (TLC), Liquid Chromatography coupled to a Mass Spectrum (LC-MS) and Nuclear Resonance Magnetic (NMR). It was expected to find a glycolipid biosurfactant, however, our spectrum shows that this biosurfactant does not have a sugar chain in its structure, but our spectra suggest that it is a triglyceride. RNA-seq based gene expression analysis identified more than 6,000 expressed genes, in which 829 are differently expressed between control (without KWO) and KWO samples. Principal component analysis indicates that these samples differ substantially at gene expression level, in which genes related to RNA metabolism and transport, ribosome biogenesis and the oxidative phosphorylation system are highly expressed in KWO samples, when compared to the control, as evidenced by enrichment analysis. By contrast, several metabolic pathways seem to be down regulated in KWO samples. The precursor enzymes to produce biosurfactants were shown in this study, having a greater expression in precursors of the metabolism pathways of lipids and fatty acids, where one of the fractions of biosurfactants is produced. These results indicate that the use of oil as carbon source to produce the biosurfactant leads to dramatic changes in gene expression of the yeast. The crude biosurfactant, F1 and F2 were also evaluated against the gram-positive bacteria *Bacillus cereus* and showed good antibacterial activity with MIC of 120, 240 and 60 µg/mL respectively and MBC of 480, 240 and 60 µg/mL, respectively.

Keywords: Residual oil, surfactant, gene expression, molecular techniques, surface tension.

1 INTRODUCTION

Biosurfactants are secondary metabolites produced by microorganisms in their stationary growth phase (Sambanthamoorthy et al., 2014; Santos et al., 2016). The production of biosurfactants was first demonstrated in 1941 (Bushnell and Haas 1941). Biosurfactants can be produced intra or extracellularly, exhibit an amphiphilic nature and have a hydrophilic and a hydrophobic fraction, which enable these compounds to ~~to~~ reduce surface tension and promote the emulsion of immiscible liquids (Kapadia and Yagnik, 2013). Biosurfactants can be grouped according to their chemical nature (phospholipids, glycolipids, lilpoptides, lipolysaccharides, polymeric biosurfactants and mycolic acids) (Ahimou et al., 2001).

Biosurfactants have attracted attention in recent years due to their wide applicability in various industrial areas. When compared to chemical surfactants, biosurfactants also draw attention because they are highly biodegradable, due to their low toxicity and stability under extreme conditions of temperature, pH and salinity (Diáz de Rienzo et al., 2015). Recently, several functions of biosurfactants have been demonstrated, including emulsifying ~~activity~~, hemolytic ~~activity~~, antiviral ~~activity~~ and antitumor activities (Gudiña et al., 2010; Rodrigues, 2011; Gomaa, 2013; Rufino et al., 2013, Gudiña et al., 2015). Another great advantage of these molecules is that some of them have antimicrobial, larvicidal and insecticidal activity, which are interesting properties for application in agriculture and in the chemical, pharmaceutical and cosmetic industries (Mnif e Ghribi, 2015; Jemil et al., 2017). Thus, identifying new biosurfactants and optimizing the methods to produce them represent a great biotechnological advance, with clear industrial applications.

The microbial group best known for producing biosurfactants are bacteria, but they are also produced by yeasts and filamentous fungi (Fontes et al., 2008). The great interest in producing biosurfactants from yeasts is that most of them are generally recognized as safe (GRAS), which allows their products to be applied in the pharmaceutical and food industries (Fontes et al., 2008). Therefore, the evaluation of the production of biosurfactant produced by yeasts has increased and several strains of non-pathogenic yeasts have been reported as producers (Faria et al., 2015; Elshafie et al., 2015; Souza et al., 2018; Eldin et al., 2019). However, it is not fully known which genes are involved in gene expression during the production of yeast biosurfactants, with this knowledge, it may be possible to improve the culture media and the substrate for fermentation, making biosurfactants more competitive on the market when compared to chemical surfactants.

Despite all the advantages of biosurfactants compared to chemical surfactants, their production cost is still expensive for an industrial scale, which makes it necessary to look for low-cost substrates. As a common and cheaper waste, used cooking oil (KWO), resulting from the food industries and household waste, contain a large amount of vegetable oils, animal fats and salt. Therefore, the high humidity and the large amount of protein present in this residue cause its rapid degradation, stimulating the growth of microorganisms (Lan et al., 2015). Thus, biosurfactants produced from KWO can reduce the cost of production and reduce the environmental stress generated by industrial and kitchen waste (Chen et al., 2018).

2 JUSTIFICATION

The production of biosurfactants using cheap substrates has some advantages, which include commercial production costs can be reduced; many cheap and renewable substrates are available; substrates are accessible in large quantities; the performance of the biosurfactant is improved; basic functional properties of the product are not changed; it is not harmful to microorganisms and all components are environmentally friendly and safe.

Thus, residual cooking oil is a common and inexpensive industrial and domestic waste that contains a wide variety of vegetable oils, animal fats and salts, causing the oil's high moisture and protein content to cause its rapid degradation and stimulation microbial growth. Therefore, biosurfactants produced from this type of oil can not only reduce the cost of production, but also alleviate the environmental pressure caused by industrial and kitchen waste.

3 GOALS

3.1 MAIN GOAL

Purify and identify the structures of the glycolipid biosurfactants produced by yeast *Wickerhamomyces anomalus* CCMA 0358.

3.2 SPECIFIC GOAL

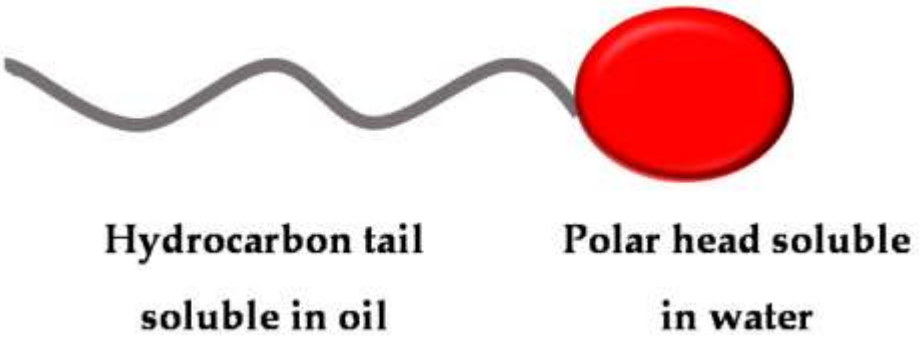
- Evaluate gene expression during the production of biosurfactant using KWO as substrate.

4 LITERATURE REVIEW

4.1 Biosurfactants

Biosurfactants are amphipathic compounds formed by a hydrophilic and a hydrophobic component (Figure 1). This characteristic gives these compounds the ability to reduce surface tension and promote the emulsion of immiscible liquids. Biosurfactants are an alternative to replace chemical surfactants produced from oil. These compounds with microbial surface activity are a group of structurally diverse molecules, produced by different microorganisms and are mainly classified by their chemical structure and their microbial origin. Biosurfactants are produced by a wide variety of bacteria and fungi. Thus, several types of structures are formed as phospholipids, glycolipids, lipopeptides, polymeric surfactants and others (COSTA, et. al., 2018).

Figure 1 – Biosurfactant molecule with hydrophilic (polar) and hydrophobic (nonpolar) fraction.



Source: *Int. J. Mol. Sci.* **2016**, *17*(3), 401; https://doi.org/10.3390/ijms17030401.

Biosurfactants have several advantages over chemically produced surfactants, such as less toxicity; greater biodegradability; better environmental compatibility; greater selectivity and specific activity in conditions of adverse temperatures, pH, salinity, use in environmental control; and finally, the ability to be synthesized from renewable raw materials (COSTA, et. al., 2018). A major problem in the production of biosurfactants is the cost involved in the process. The carbon source is classically a significant part of the costs of producing biosurfactants. However, this can

be significantly reduced by using alternative sources of nutrients, which are easily available and inexpensive.

The use of industrial waste as an energy source to produce biosurfactants is an attractive alternative to reduce production costs, making the process viable (AL-BAHRY, et. Al., 2013). However, the selection of residual substrates involves the difficulty of finding a residue with the right balance of nutrients to promote the growth of the microorganism and the production of metabolites. Agro-industrial residues with a high content of carbohydrates, lipids and proteins are attractive to be used as substrate; they usually have the necessary nutrients for that (AKPINAR & UREK, 2012).

The use of cheaper and renewable substrates from various industries, such as agriculture (sugars, molasses, vegetable oils, oil residues, starch, whey), distilleries, animal fat and petroleum industries, has been reported and reviewed by researchers (MAKKAR, CAMEOTRA AND BANAT, 2011). However, large-scale production for most microbial surface-active agents has not reached a satisfactory economic level due to their low yields. In addition, a high cost for further processing is required to recover and purify microbial surfactants. Such obstacles can be overcome by isolating potential producers of biosurfactants who can use renewable substrates to increase the quality and quantity of biosurfactants (SMYTH, et al., 2010).

The production of biosurfactants using cheaper substrates has some advantages and disadvantages. As advantages, they include commercial production costs can be reduced; many cheap and renewable substrates are available; substrates are available in large quantities; the performance of the biosurfactant is improved; basic functional properties of the product are not changed; it is not harmful to microorganisms and all components are environmentally friendly and safe. As disadvantages are included: the substrates can contain unwanted compounds; the processing or treatment of the substrates is necessary to use them as carbon, nitrogen, or an energy source; special purification techniques need to be employed to obtain purified products; a continuous supply of raw materials with the same composition may vary; raw substrates can be very specific for different organisms and a large availability of raw substrates is essential, as it can be difficult to obtain a continuous supply for an industrial process (BANAT, et. al., 2014).

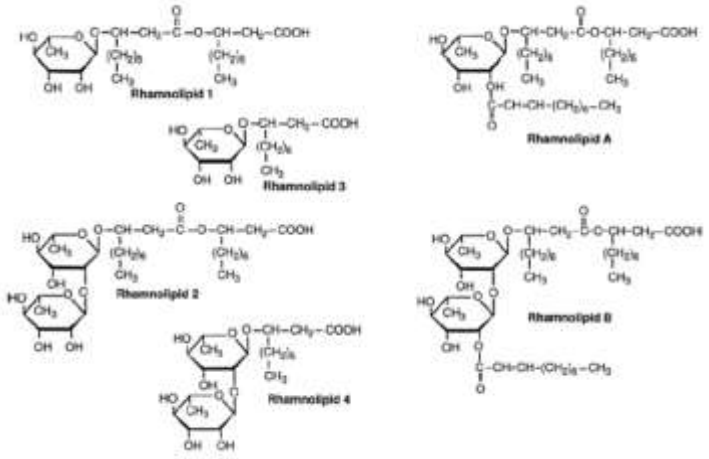
4.2 Classification of biosurfactants

These surfactants, depending on their origin, can be synthetic (surfactants) or natural (biosurfactants), and biosurfactants (BS) are composed of amphiphilic molecules with hydrophilic and hydrophobic fractions (López-Prieto et al., 2019). The polar portion can be ionic (cationic or anionic), non-ionic or amphoteric (possess both positive and negative charges depending on the environment in which they are present) and the non-polar portion commonly comprises a hydrocarbon chain (Santos et al, 2017). Like all surface-active molecules, biosurfactants contain one or more lipophilic and hydrophilic moieties. The lipophilic moiety may be a protein or peptide with a high proportion of hydrophobic side chains, or it may be a fatty acid hydrocarbon chain having 10 and 18 carbon atoms, although higher molecular weight fatty acids have been reported. The hydrophilic portion can be an ester, hydroxyl, phosphate or carboxylate group, or a sugar carbohydrate (CAMPOS, et. Al., 2013). These molecules have different structures and are broadly classified into low molecular weight biosurfactants (for example, glycolipids, lipopeptides and phospholipids) and high molecular weight (for example, lipoproteins, lipopolysaccharides, complex biopolymers, etc.) (ROSEMBERG AND RON, 1999).

4.2.1 Glycolipids

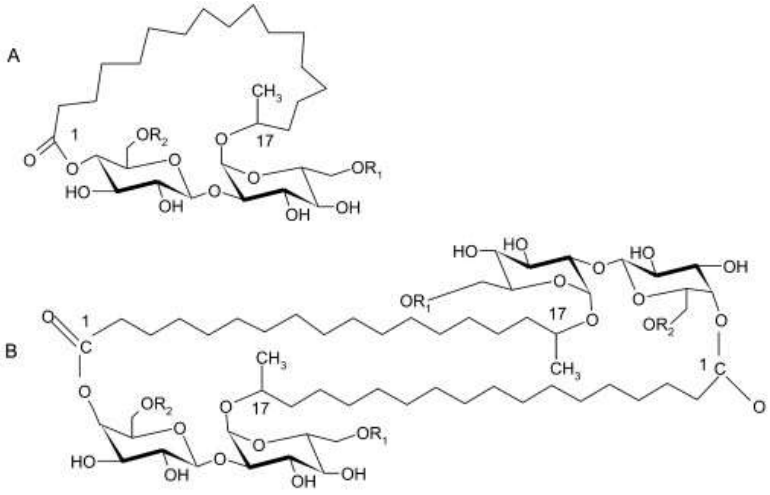
Glycolipids are one of the most popular biosurfactants, with ramnolipids (Figure 2), soforolipids (Figure 3) and trehalolipids (Figure 4) the most well-known. Structurally, they comprise a fatty acid in combination with a carbohydrate portion and correspond to a group of compounds that differ in relation to the nature of the lipid and carbohydrate portion. As a result of the type of carbohydrate diversity, glycolipids can be subdivided into ramnolipids, trehalolipids, soforolipids, cellobiose lipids, manosylerythritol, lipomannans and lipoarabinomanans, diglicosil diglycerides, monoacylglycerol and galactosyl diglycerides.

Figure 2 – Illustration of the chemical structure of rhamnolipid surfactants.



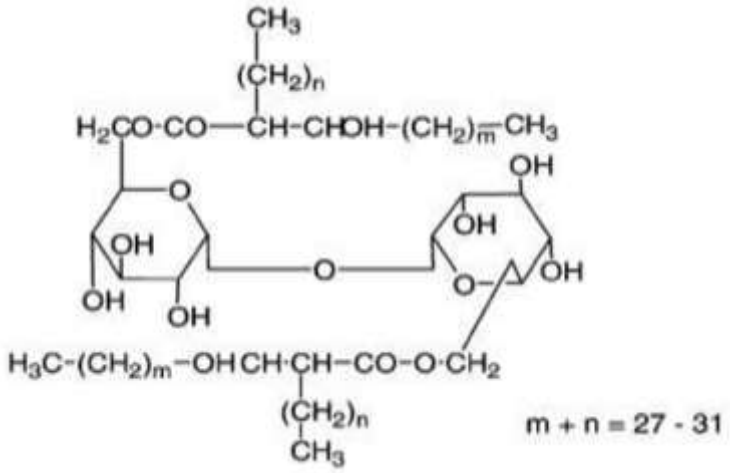
Source: *Progress in Understanding Cystic Fibrosis*, **2017,** 127.

Figure 3 – Illustration of the chemical structure of soforolipid surfactants.



Source: *Extracellular Glycolipids of Yeasts*, **2014**, 1-13.

Figure 4 – Illustration of the chemical structure of trehalolipid surfactants.



Source: Production and characterization of biosurfactant from bacterial isolates (Doctoral dissertation, Netaji Subhas Institute of Technology).

Rhamnolipids are the most studied biosurfactants. Its production by *Pseudomonas aeruginosa* was first described by Jarvis and Johnson (1949). Subsequently, several bacterial strains producing ramnolipids were isolated. Trehalolipids represent a broad group of glycolipids consisting of a trehalose disaccharide linked to mycolic acids, which comprises long chain branched fatty acids. They are produced mainly by Gram-positive bacteria, with a high GC content, such as *Mycobacterium, Nocardia* and *Corynebacterium*, and differ in their structure, size and degree of saturation. Soforolipids are mainly produced by yeast strains such as *Candida bombicola, Candida magnoliae, Candida apicola* and *Candida bogoriensis* when grown on carbohydrates and lipophilic substrates.

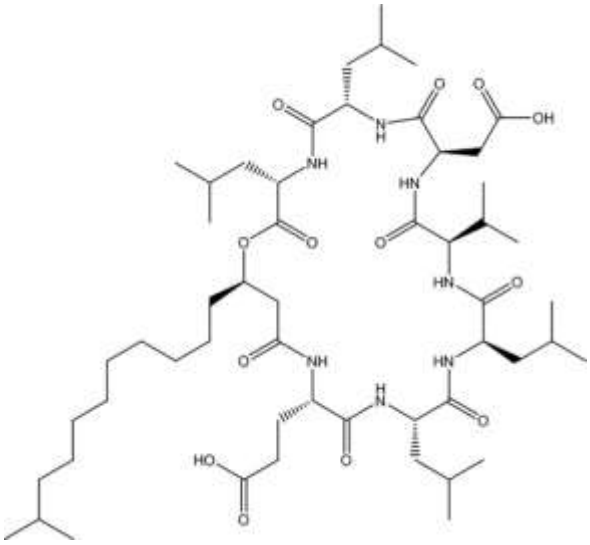
Microbial glycolipids exhibit many interesting functional properties, such as the ability to reduce surface and interfacial tension, emulsification and demulsification capabilities, foaming power, solubilization and mobilization skills and pore forming capabilities. In addition, they are recognized for their interesting physicochemical properties, including stability in the face of extreme pH, salinity and temperature conditions. Thus, they have the potential to be used in the environmental field to improve the solubility, mobility and biodegradation of hydrocarbons. They

are also potential candidates for biomedicine and therapy because of their antimicrobial, hemolytic, antiviral, anti-cancer and immunomodulatory activities. Furthermore, as a result of a broad spectrum of emulsion capacity and anti-adhesive activity, they have the potential to be used in the food industry. In the field of agriculture, they promote the inhibition of certain phytopathogenic fungi, algae and insect larvae.

4.2.2 Lipopeptides

Lipopeptides are surface active microbial compounds produced by a wide variety of bacteria and fungi (Figure 5). They are characterized by highly structured diversity and can decrease surface and interfacial tension at the surface and interface, respectively. Surfactin, iturin and fengicin are among the most studied lipopeptides. Usually, lipopeptides are produced by aerobic microorganisms such as: bacteria, fungi, yeasts and actinomycetes. Lipopeptide surfactants are naturally produced as a mixture of several macromolecules belonging to the same family or class (INÈS & DHOUHA, 2015).

Figure 5 – Illustration of the chemical structure of lipopeptide surfactants.



Source: In Biobased Surfactants, **2019,** pp. 205-240. https://doi.org/10.1016/B978-0-12-812705-6.00006-X.

4.2.3 Phospholipids, neutral lipids and fatty acids

Phospholipids, neutral lipids and some fatty acids are components of cellular structures and have superficial activity (SATPUTE et al., 2010). Some examples of this type of biosurfactants

are gramicidin and polymyxin (DESAI & BANAT, 1997). They are biopolymers consisting of a polysaccharide skeleton to which the fatty acid side chains are covalently attached (SATPUTE et al., 2010). Fungi, yeasts and bacteria have the ability to grow on hydrophobic substrates such as alkanes, and to secrete large amounts of phospholipids, fatty acids or neutral lipids to facilitate the absorption of the carbon source (SHEKHAR et al. 2015).

4.3 Biosurfactant-producing microorganisms

The function of the biosurfactant in a microbial producing cell is involved in the emulsification of water-insoluble substrates, which helps the cell to survive in a hydrophobic medium (MORIKAWA, HIRATA AND IMANAKA, 2000). In addition, biosurfactants have been shown to be involved in cell adhesion, providing greater stability under hostile environmental conditions and virulence, in cell desorption to find new habitats for survival, in antagonistic effects in relation to other microorganisms in the environment (MIDDELBERG AND DIMITRIJEV - DWYER, 2011).

Microorganisms use a set of carbon and energy sources for growth. The combination of carbon sources with insoluble substrates facilitates intracellular diffusion and the production of different substances. Microorganisms (yeasts, bacteria and some filamentous fungi) are capable of producing biosurfactants with different molecular structures and surface activities (COSTA, et. al., 2018).

4.3.1 Bacteria

Bacteria are the main group of microorganisms that produce biosurfactants, these microorganisms can synthesize them from hydrocarbons, such as glucose, sucrose, glycerol or ethanol and can either be excreted or remain intracellularly (DESAI E BANAT, 1997). Bacteria play an important role in the production of biosurfactant, with *Pseudomonas* being the predominant genus (SHEKHAR et al. 2015).

Lactobacillus species synthesize less biosurfactants when compared to *Pseudomonas aeruginosa* but are part of a safe source of biosurfactants because they are considered safe microorganisms and are already used in the manufacture of some foods (GUDIÑA et al., 2011).

4.3.2 Yeast

Evaluation of yeast biosurfactant production has increased in recent years, and several strains have been reported as promising biosurfactant producers due to their high yields and high substrate conversion rates. Among them are species belonging to the genera *Candida, Starmerella, Pseudozyma* and *Yarrowia* (FARIA, et. al., 2015 & ELSHAFIE, 2015).

The great advantage of using yeasts in the production of biosurfactants is that most of these species are recognized as GRAS (Generally Recognized as Safe), such as, for example, *Yarrowia lipolytica, Saccharomyces cerevisiae* and *Kluyveromyces lactis*, which allows the application of their products in the pharmaceutical and food industry (FONTES, AMARAL E COELHO 2008).

An example of a biosurfactant produced by yeasts is the lipid mannosileritritol (MEL), a glycolipid that has excellent interfacial property and exhibits high antimicrobial activity against Gram-positive bacteria. This glycolipid is used in the cosmetics industry, being used in dry skin moisturizers and in products for repairing hair damage (MORITA, et al., 2013).

4.3.2.1 *Wickerhamomyces anomalus*

Wickerhamomyces anomalus (*W. anomalus*) is often found as an environmental colonizer that can be isolated from organic substances, including vegetables, fruits and soil (KAMOSHITA et al., 2015). *W. anomalus* CCMA 0358 has been identified as a producer of biosurfactants growing in a culture medium containing glucose (1 g/L) and olive oil (20 g/L) as carbon sources. This strain produced 2.6 g of biosurfactant per liter after 24 hours of growth. The crude biosurfactant reduced the surface tension of water to values around 31 mN/m, and its critical micelle concentration was 0.9 mg/mL. (SOUZA, et. Al., 2017).

4.3.3 Filamentous fungi

Studies have shown the production of biosurfactants also by fungi. Marine fungi were isolated from sea sponges, with promising results from all isolates of *Aspergillus ustus* to produce biosurfactants (KIRAN, et al., 2009). Marine fungi live in a stressful environment, without light, high pressure, or other types of mechanical stress. Their ability to survive in different environmental conditions makes them attractive for isolating new molecules, such as biosurfactants. Compounds with surfactant properties belonging to the genera *Penicillium* and *Talaromyces* were isolated (NICOLETTI & TRINCONE, 2016).

4.4 Industrial applications of biosurfactants

Biosurfactants can be produced in different amounts by various microorganisms. The diversity of its chemical structures leads to a variety of functions that include the reduction of surface tension and, thus, the reduction of interfacial tension. They also increase surface areas, which have been shown to be useful in water-insoluble hydrophobic compounds. Their ability to disperse or dissolve hydrophobic compounds has made them useful in a variety of industries, such as the pharmaceutical, food and energy industries. They are also able to detect quorum with the ability to initiate cell signaling. These substances can bind heavy metals. They can be pathogenic for bacteria and can form biofilms (ABACHA, SANDERSON AND RAHMAN, 2016).

4.4.1 Environmental applications

Most persistent contaminants have low water solubility and, therefore, the addition of emulsifiers can increase the bioavailability of the contaminants for metabolizing microorganisms. By reducing the surface and interfacial tension between liquids, solids and gases, biosurfactants can easily disperse as emulsions so that the process of ingesting contaminants as a nutrient for microorganisms can be improved (BANAT, DIAZ DE RIENZO & QUINN, 2014).

Usually, the degradation time and the adaptation time for microorganisms have been shortened using biosurfactants. Many contaminated sites in the Middle East have been remedied by adding biosurfactant to the contaminated environment in addition to other nutrients. These sites represented soil and sand contaminated by heavy hydrocarbons, mainly of industrial origin. Kosaric (2001) demonstrated that bioremediation accelerated when glycolipid biosurfactants were added (0.5 kg / ton of soil) to the nutrient applied to the soil.

Many studies can be found using biosurfactants in environmental applications. El-Sheshtawya et al., 2016, used the bacterium *Bacillus licheniformis* and the yeast *Candida albicans*, in which the potential application of these species to microbial oil recovery was investigated. Oil recovery was 16.6 and 8.6% by weight for bacterial and yeast strains, respectively, after application to a sand column designed to stimulate oil recovery. In the same application area, Ivshina et al., 2016, evaluated the removal of polycyclic aromatic hydrocarbons in the soil using biosurfactants produced by *Rhodococcus ruber* in soil columns with model mixtures of the main oil constituents. The UV spectra for the soil before and after treatment with biosurfactant were obtained and its applicability for differentiated analysis of polycyclic aromatic hydrocarbons and changes in the

concentration of polycyclic aromatic sulfur heterocycles in remedied soil was demonstrated. The A254 nm / A288 nm ratios revealed that biosurfactants increased the biotreatment capacity of soils contaminated with polycyclic aromatic hydrocarbons.

In another study by Xia et al., 2011, biosurfactants from three bacteria were compared for their bioremediation activity and all showed good promise in oil recovery, even when used at extreme levels of pH, temperature, metal ions and salinity. The three bacteria used were P. aeruginosa, *B. subtilis* and *Rhodococcus erythropolis*. *P. aeruginosa* had the highest emulsification rate of 80%. These highly efficient bioremediation agents are being used to clean up soil contaminated by hydrocarbons and heavy metals, as it has been found that only about 30% of oil contaminants can be removed by conventional primary and secondary techniques.

4.4.2 Petrochemical industry

The presence of paraffin in crude oil results in increases in the solidification point and oil viscosity, which influences the transport capacity and the operational safety of the pipelines, thus making it difficult to extract oil (SAKTHIPRIYA, DOBLE & SANGWAI, 2015). Conventional mechanical, thermal and chemical methods to clean the paraffin deposited on the surface of the pipes have the disadvantages of the high difficulty of operation, potential damage to formation (ETOUMI, 2007) and the use of expensive and dangerous chemicals. In recent decades, microbial methods have proven effective in mitigating and preventing paraffin deposition and are considered an alternative to conventional methods (ZHANG, et al., 2012).

Microbial paraffin removal is a technique that uses microorganisms or their metabolic by-products (eg, biosurfactants and paraffin solvents) for the control and removal of paraffin deposition from oil wells and production facilities. This method is economical and ecological (LAZAR, et al., 1999). Some researchers, such as Rana et al (2010) and Zhang et al (2012), reported that the use of microorganisms and their metabolic products can mitigate the deposition of paraffin in oil wells and surface flow lines. The main areas of application include the United States, Venezuela, Indonesia and China. In the Daqing, Jianghan, Liaohe, Zhongyuan and Jidong oil fields in China, microbial treatment has resulted in obvious increases in oil production and is considered a cost-effective approach (ZHANG, et al., 2014).

The mechanisms by which bacteria are able to mitigate and remove paraffin deposition have been described by Hao et al (2004): (a) the microbial degradation of paraffin long-chain n-

alkanes is converted to short-chain n-alkanes, which improves the solubility of paraffinic oil; (b) alteration of the paraffin's physical properties by microbial products, including fatty acids, alcohols, emulsifiers and biosurfactants; and (c) formation of biofilms by the adsorption of microorganisms on the surface of the paraffin, which prevents the crystallization and deposition of paraffin. The microbial treatment for the control of paraffin deposits must be based on bacterial products which are paraffinic oil, non-pathogenic, naturally occurring, and the by-products of which are alcohols, gases, acids, emulsifiers and biosurfactants, which cause changes remarkable in the physicochemical properties of paraffinic oil and the well-bore area (ETOUMI, 2007).

4.4.3 Agriculture

The potential of biosurfactants for disease control in plants has been widely reported in recent years (BORAH et al., 2015, LAHKAR et al., 2015). Among all types of biosurfactants reported to date, most research has been done on surfactin produced by *Bacillus* sp. and rhamnolipids produced by *Pseudomonas* sp. (PERFUMO et al., 2006). Rhamnolipids are effective in the control of pathogenic fungi and it was preliminarily established by Stanghellini and Miller (1997), where the *P. aeruginosa* ramnolipid producing strain can lead to the lytic effect of spores from zoosporic plant pathogens. It was mentioned that the rhamnolipid in contact with the zoospores leads to cessation of motility, followed by rupture of the plasma membrane and lysis of the entire spore.

4.4.4 Pharmaceutical applications

According to Gudiña et al., 2013, biosurfactants emerged as promising molecules due to their structural novelty, versatility and diverse properties potentially useful for many therapeutic applications. Mainly due to their surface activity, these molecules interact with the cell membranes of various organisms and / or with the surrounding environments and, therefore, can be seen as possible cancer therapies or as constituents of drug delivery systems. Some types of microbial surfactants, such as lipopeptides and glycolipids, have been shown to selectively inhibit the proliferation of cancer cells and disrupt cell membranes, causing their lysis via apoptosis. In addition, biosurfactants as drug delivery vehicles offer commercially attractive and scientifically new applications.

Other works can be found in the literature in this specific application, such as the one published by Akiyode et al., 2016, which concluded that biosurfactants were more effective in

retarding the growth of the tested cancer cell lines and, therefore, may be potential candidates for use in human cancer therapy. The physical-chemical characteristics of biosurfactants suggest that their mechanism of action may be due to activity on the cell membrane.

4.4.5 Food industry

Surfactant compounds exhibit a variety of functions in the food industry, especially as emulsifiers, foaming agents, or humectants, in addition to promoting the solubility of different food ingredients (GUDNA AND RODRIGUES, 2020). Emulsification plays an important role in the consistency and texture of many food products. (MCCLEMENTS et al. 2017). Many natural and processed food products are emulsions. Examples of emulsions include butter, margarine or fat-based products, milk, sauces, mayonnaise, or ice cream, among others (KRALOVA AND SJÖBLOM 2009).

Biosurfactants also have great potential for application in various sectors of the food industry not restricted to food processing (GUDNA AND RODRIGUES, 2020). Among the most interesting properties of these compounds, their antimicrobial and anti-adhesive activities can represent powerful weapons to ensure food safety. Several biosurfactants, such as soforolipids, raminolipids and surfactin, have proven to act as antimicrobial agents against different bacteria, yeasts, fungi, algae and viruses (RODRIGUES et al. 2006; NITSCHKE AND COSTA, 2007; RODRIGUES, 2011).

In addition to their full potential, biosurfactants have been suggested as valuable molecules for the synthesis of nanoparticles and liposomes (Kiran et al. 2011). Recently, a growing interest in processes mediated by biosurfactants has been reported, mainly due to their potential role in the synthesis of silver nanoparticles and NiO nanorods (XIE et al. 2006; PALANISAMY, 2008). Reddy and collaborators (2009) stabilized the synthesis of silver nanoparticles with surfactin. Ramnolipids were also evaluated for their effect on the synthesis and stabilization of nanozirconia particles (Biswas and Raichur 2008). Biosurfactants have been accepted as safe, versatile and useful for several applications, including the development of nanosolutions for the food industry (GUDNA AND RODRIGUES, 2020).

4.5 Ecofriendly and cheaper substrates to produce biosurfactants

Over the past decade a wide variety of cheap and renewable industrial waste has been discovered to be used in the production of biosurfactant. Among them, food and agribusiness residues stand out, thus bringing an effective cost cutting strategy together with much-needed waste management (Banat et al. 2014; Satpute et al. 2017; Singh, et al, 2018). The use of industrial waste to produce valuable compounds has assumed importance in recent times, not only in the economy of any commercial production process, but also in the establishment of a sustainable environment, an effort for an effective management of the new waste generated (Patil and Rao, 2015). However, it is not limited only to the cost of raw materials, but the availability, stability and variability of each component are also critical factors to be considered, as they all play a critical role in the final selection and formulation of any substrate to produce biosurfactant (Singh, et al, 2018).

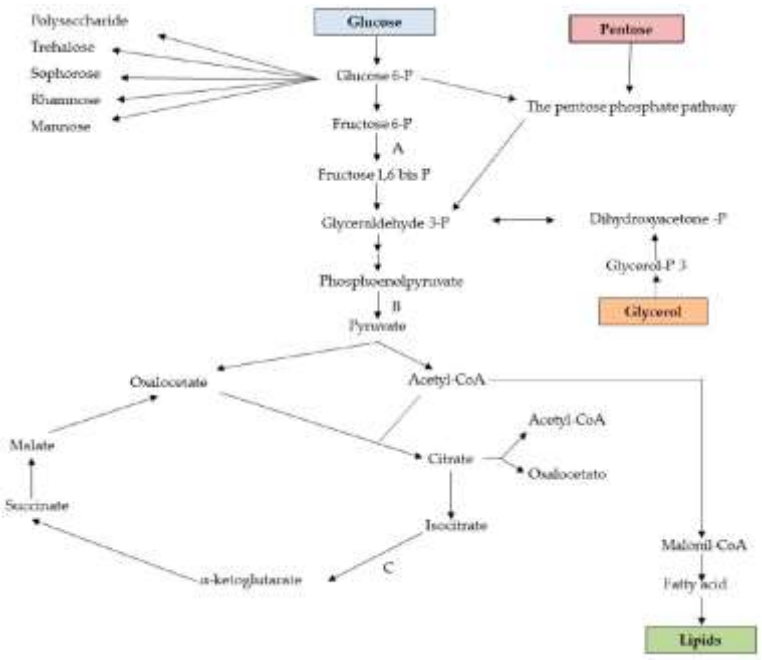
The most recent and prominent low-cost waste materials explored as a substrate for the production of biosurfactant were: Agro-Industrial and Food Waste: produced by *Bacillus licheniformis* using orange peel (Kumar et al., 2016); produced by *Halobacteriaceae archeon* using banana peel (Chooklin et al., 2014); produced by *Bacillus pumilis* using potato peel (Sharma et al., 2015); produced by *Candida tropicalis* using cane bagasse, moringa residue and cassava residue (Rubio-Ribeaux et al., 2017); produced by *Cunninghamella phaeospora* using corn steeping liquor (Lins et al., 2016). Animal waste: produced by *Pseudomonas gessardii* using slaughterhouse waste (Ramani et al., 2012); produced by *Nocardia higoensis* using animal fats (Patil et al., 2016); produced by *Aneurinibacillus migulanus* using fish processing residues Sellami et al., 2016). Agro-industrial and grinding waste: produced by *Bacillus subitilis* using waste from olive mills (Moya - Ramírez et al., 2016); produced by *Pseudomonas aeruginosa* using waste from the palm oil industry (Radzuan et al., 2017); produced by *Bacillus pseudomycoides* using residues from the soy oil industry (Li et al., 2016). Residues from industrial and cooking oil: produced by *Pseudomonas aeruginosa* using residues of coconut oil for frying (George and Jayachandran, 2013); produced by *Candida lipolytica* using residues from cooking oil (Lan et al., 2015); produced by *Wickerhamomyces anomalus* using kitchen waste oil (Fernandes, et al, 2020).

4.6 Metabolic Pathways of Biosurfactant Production

The biosynthesis of secondary metabolites, such as glycolipids, occurs mainly under conditions of lack of nitrogen or a high C/N ratio. For the microorganisms, while the primary metabolism genes are distributed throughout the genome, the genes involved in the synthesis of secondary metabolites are generally organized in clusters, facilitating activation and regulation (Demain, 1998). This organization of clusters can be advantageous for the horizontal transfer of genes between different species and can guarantee the transfer of complete biosynthesis pathways (Jezierska, Claus and Van Bogaert, 2018).

Hydrophilic substrates are used mainly by microorganisms for cellular metabolism and the synthesis of the polar portion of a biosurfactant, while hydrophobic substrates are used exclusively to produce the hydrocarbon portion of the biosurfactant (Desai and Banat, 1997). Several metabolic pathways are involved in the synthesis of precursors to produce biosurfactants and depend on the nature of the main sources of carbon used in the culture medium. For example, when carbohydrates are the only carbon source to produce a glycolipid, the carbon flow is regulated so that the lipogenic pathways and the formation of the hydrophilic fraction through the glycolytic pathway are suppressed by microbial metabolism (Figure 6) (Haritash and Kaushik, 2009).

Figure 6 – Intermediate metabolism related to the synthesis of biosurfactant precursors using carbohydrates as a substrate. Enzymatic keys for carbon flow control: (A) phosphofructokinase; (B) pyruvate kinase; (C) isocitrate dehydrogenase.

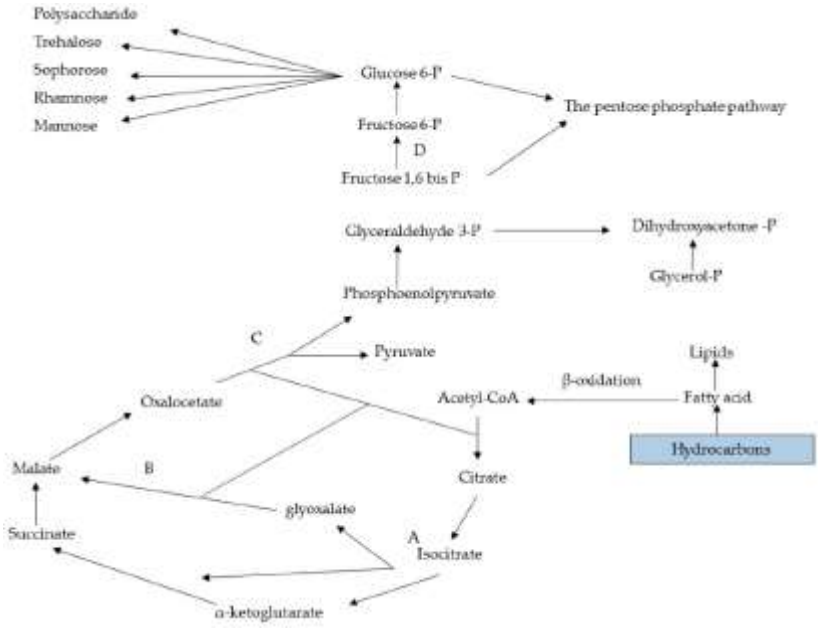


Source: *Int. J. Mol. Sci.* **2016**, *17*(3), 401; https://doi.org/10.3390/ijms17030401.

A hydrophilic substrate, such as glucose or glycerol, is degraded to form intermediates in the glycolytic pathway, such as glucose 6-phosphate, which is one of the main carbohydrate precursors found in the hydrophilic fraction of a biosurfactant. To produce lipids, glucose is oxidized to pyruvate through glycolysis and pyruvate is then converted to acetyl-CoA, which produces malonyl-CoA when joined with oxaloacetate, followed by conversion to fatty acid, which is one of the precursors of synthesis of lipids (Hommel and Huse, 1993). When a hydrocarbon is used as a carbon source, however, the microbial mechanism is mainly directed towards the lipolytic pathway and gluconeogenesis (the formation of glucose through different hexose precursors), thus allowing its use in the production of fatty acids or sugars. The gluconeogenesis pathway is activated to produce sugars. This pathway consists of the oxidation of fatty acids through β-oxidation to acetyl-CoA (or propionyl-CoA in the case of odd-chain fatty acids). Starting with the formation of acetyl-CoA, the reactions involved in the synthesis of polysaccharide precursors, such as glucose 6-phosphate, are essentially the reverse of those involved in glycolysis. However, the reactions catalyzed by pyruvate kinase and phosphofructokinase-1 are irreversible. Thus, other enzymes exclusive to the gluconeogenesis process are needed to avoid such reactions. Figure 7 illustrates the main reactions to the formation

of glucose-6-phosphate, which is the main precursor of polysaccharides and disaccharides formed to produce the hydrophilic fraction of glycolipids (Tokumoto, et al, 2009).

Figure 7 – Intermediate metabolism related to the synthesis of biosurfactant precursors using hydrocarbons as substrate. Key enzymes: (A) isocitrate lyase; (B) malate synthase; (C) phosphoenolpyruvate; (D) fructose-1.



Source: *Int. J. Mol. Sci.* **2016**, *17*(3), 401; https://doi.org/10.3390/ijms17030401.

According to Sydatk and Wagner (1987), the biosynthesis of a surfactant occurs through four different routes: (a) synthesis of carbohydrates and lipids; (b) synthesis of the carbohydrate half, while the synthesis of the lipid half depends on the length of the carbon substrate chain in the medium; (c) synthesis of the lipid half while the synthesis of the carbon half depends on the substrate used; and (d) synthesis of the carbon and lipid moieties, which are dependent on the substrate. Therefore, the length of the n-alkane chain used as a carbon source alters the biosynthesis of a surfactant (SANTOS, et al., 2016).

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SECTION 2

ABSTRACT

Tea (*Camellia sinensis*) is one of the world's most important beverage crops and provides numerous secondary metabolites that account for its rich taste and health benefits. In *in vitro* cultures, numerous losses due to bacterial contamination can occur, but several studies indicate that endophytes have a positive effect on plants. Therefore, this study shows the population of bacteria found in two varieties of tea tissue culture, using the metagenomic technique. In total, 17 bacterial DNA were identified, 10 of these species were identified in both varieties, including *Klebsiella pneumoniae*, which can fix atmospheric nitrogen into a form that can be used by plants, thus are called associative nitrogen fixers or diazotrophs, *Cronobacter sakazakii* often found associated with plants and rhizosphere and is related to solubilization of mineral phosphate and production of indole acetic acid and *Paenibacillus* spp. where many have proven to be important for agricultural and horticultural, industrial and medical applications. This study is the first to correlate microorganisms associated with *in vitro* cultivation of *C. sinensis*.

Keywords: Bacterial endophytes, in vitro culture, metagenomics.

1 INTRODUCTION

Tea is the second most popular drink in the world and offers a wide range of health benefits, such as reducing cardiovascular mortality and treating digestive disorders (Begas et al., 2017). Tea consumption has a history of almost 5,000 years (Yang and Hong, 2013). *Camellia sinensis* is a member of the *Theaceae* family, which was an endemic species in southwest China and is now cultivated worldwide (Taniguchi, et al., 2014). In the last decade, world tea production increased by 66% in planted area and reached 5.95 million tons in 4.1 million hectares in 50 tea producing countries (FAO, 2020). The combination of genetic variation, environmental factors and various modes of tea processing has created a huge variety of tea products with diverse palatability, such as bitter, astringent and sweet flavors, to meet consumer demand worldwide (Wei et al, 2018).

Under commercial conditions, losses can be severe during *in vitro* rooting and acclimatization of tea plants. These problems cannot be overcome by manipulating culture media or other growing conditions, and a possible explanation for the variation could be the presence of different populations of endophytic bacteria (Quambusch, et al, 2014). Endophytes are bacteria or fungi that, for part of their life, can live inside plant tissue without triggering symptoms of disease (Petrini 1991). Endophytic bacteria are frequently observed in plant cultures *in vitro*, both in commercial laboratories and in scientific studies (Leifert et al. 1991), and frequently affect the *in vitro* propagation of plants (Ulrich et al. 2008). A frequently used method is the addition of antibiotics to the culture medium to suppress bacterial growth (Asif et al. 2013; Bohra et al. 2014).

Herman (1989) presented the idea that non-pathogenic bacteria or even growth promoters can become harmful in the special growth conditions of the culture *in vitro*. In contrast, several studies indicate that endophytes have a positive effect on plants, for example, through the biosynthesis of growth-stimulating phytohormones, increased availability of nutrients and induced resistance to pathogens (Goh and Vallejos 2013). In a study of strawberry tissue cultures, some isolated endophytic bacteria showed growth-promoting effects of plants during the greenhouse acclimatization process (Dias et al. 2008).

Therefore, it is important to identify bacteria in the tissue culture of *Camellia sinensis* and use this knowledge to develop strategies to potentially influence the structure of plant propagation *in vitro*. As far as we know, there are no studies of bacteria associated with *in vitro* cultivation of *C. sinensis*.

2 JUSTIFICATION

Tea is a non-alcoholic beverage widely consumed in the world, with essential economic and health benefits, and most of its production is concentrated in eastern countries. The state of California has been conducting tea production trials since 1964, indicating the possible growth of this crop in the USA, but growing it is still a challenge.

Using tissue culture, it is possible to minimize the environmental and adaptive interference of tea plants, in addition to being able to produce healthy plants without chemical additives. Knowing the microbiota that lives in this culture, it will be possible to determine in advance the possible benefits and harms of the bacteria present.

3 GOAL

- Identify bacteria present in tea tissue culture (*Camellia sinensis*) using the metagenomics technique.

4 LITERATURE REVIEW

4.1 Camellia sinensis

Camellia sinensis is the species of plant whose leaves and leaf buds are used to make tea. It is in the genus *Camellia*, a genus of flowering plants in the family *Theaceae*. *Camellia sinensis* is native to mainland China, south and southeast Asia, but today it is grown worldwide in tropical and subtropical regions. It is a perennial shrub or small tree that is usually trimmed to less than two meters (six feet) when grown for its leaves. It has a strong main root. The flowers are yellow-white, 2.5-4 cm in diameter and 7 to 8 petals. The tea drink is an infusion of dried leaves of *Camellia sinesis* (Namita, Mukesh and Vijay, 2012).

The varieties of cultivated tea plants belong mainly to two main groups: *Camellia sinensis* var. *sinensis* (CSS; Chinese type) and *Camellia sinensis* var. *assamica* (CSA; type assam), being the first to cultivate more widely distributed in China and in the world. The two types of tea plants have different characteristics. CSS is a slower-growing shrub with a small leaf and can withstand colder climates, while CSA is a fast-growing shrub with large leaves and a high sensitivity to cold. CSA, therefore, is mainly grown in hot tropical areas, distinct from the broader CSS cultivation geography (Ming and Bartholomew, 2007). In agricultural practice, CSS can be grown in high-latitude areas to produce quality green tea, while CSA is generally processed into black tea (Willson and Clifford, 2012). Most of today's elite tea plant cultivars in China (~67%) belong to CSS (Yang and Liang, 2014).

Tea contains about 4000 bioactive compounds, a third of which is contributed by polyphenols (Tariq, et al, 2010). Other compounds are alkaloids (caffeine, theophylline and theobromine), amino acids, carbohydrates, proteins, chlorophyll, volatile organic compounds (chemicals that readily produce vapors and contribute to the odor of tea), fluoride, vegetable aluminum, minerals and trace elements (Cabrera, et al 2003). The great abundance of health-promoting metabolites in tea (*Camellia sinensis*) makes it one of the most popular drinks worldwide (Xiao, et al, 2020).

Due to the monoculture nature of tea cultivation, which covers a large area, plantation ecosystems are vulnerable to attack by many pests and diseases, requiring their management using agrochemicals (Zhang, et al. 2017). Plant-associate microorganisms can help to implement tea cultivation practices that are ecologically compatible with agriculture and sustainable to improve

productivity and stimulate the production of improved bioactive compounds (Bora, et al, 2021). Previous studies have shown that tea plants host diverse populations of bacteria with a different distribution pattern that can help improve their culture and productivity (Hu et al. 2006; Nath et al. 2013).

4.2 Plant-associate bacteria

An abundance of multidimensional dynamic interactions occurs between plants and microorganisms in coevolution in the regions of the rhizosphere, philosopher and endosphere (Pacheco and Segrè 2019). These microbial associations help to increase the strength of the host plant and allow them to develop and adapt in different habitats (Hassani et al. 2018). Beneficial microorganisms can stimulate plant growth through a wide range of mechanisms, including nutrient absorption, hormone production, mineral solubilization and increased ability to resist tolerance to biotic and abiotic stress (Meena et al. 2017). Thus, the plant's microbiome is a key determinant of plant health and productivity and has attracted a lot of scientific attention in recent years (Fadiji and Babalola 2020).

Bacteria-host plant interactions are classified as commensalistic, mutualistic or antagonistic (Vacheron et al. 2013). Most of the contact between plants and bacteria occurs in the root zone, where a wide variety of bacteria live, where bacterial and plant metabolites mediate the establishment of contacts and where penetration into the roots occurs. Bacteria can also enter plants through leaves, flowers, stems and cotyledons. A frequent result is the colonization of the entire plant (Compant et al. 2008). Bacteria can be transmitted through generations of plants, both vertically by means of generating organs, and horizontally by vegetative propagules (Partida-Martinez and Heil 2011).

The plant is not only a hotspot for phytopathogens, but also the native habitat for non-pathogenic microorganisms, including fungi and bacteria (Vorholt, 2012). The plant also serves as an ecological niche for associated microorganisms that can establish interactions with each other and with the host, forming a set of species that is often referred to as a "holobiont" (Hassani et al., 2018). It has also been reported that the plant's microbiota influences the formation of secondary metabolites in the plant and alters sensory perception (Schmidt et al., 2014).

Plant endophytes can live during all or part of the life cycle within the living tissue of plants (Wilson 1995). Endophytes can affect the plant's physiological processes, to an extent not unlike

the plant's genotype (Sessitsch et al. 2012), as a source of metabolites similar or different from those produced by plants (Ludwig-Müller 2015). Some endophytes are known to be highly specific to the host genotype, but others have a wider range of potential hosts (Hardoim et al. 2011).

Plant-associated bacteria form epiphytic and endophytic populations in all parts of plants (Turner et al. 2013), including meristematic cells (Pirttilä et al. 2000) or pollen (Madmony et al. 2005). These colonizing populations of bacteria and other microorganisms are recognized as the host's microbiome (Turner et al. 2013). Thus, plants in their natural environment are holobionts (Rosenberg et al. 2010), that is, they are a grouping of a host and many other species that live in or around it, which together form a distinct ecological unit. This cohabitation can extend the life potential of the partners (Rout et al. 2013).

Plants react defensively to pathogenic microorganisms, but the plants can also stimulate those microorganisms that are beneficial to their own survival (Oldroyd, 2013). Several recognition mechanisms have been discovered, but the result depends on both, signals released by bacteria and plant receptors (Carvalho et al. 2016).

The benefits may vary depending on the specific characters of the bacteria and the interactions between plants and bacteria. Some bacteria can produce not only all the hormones produced by plants (Friesen et al. 2013), but also other growth regulators, unusual for plants, that can affect plant morphogenesis and development. Aids that are produced by many bacteria affect the development of the root system, thereby increasing the absorption of nutrients and water from the soil (Patten and Glick 2002). Bacteria produce plant growth regulators and stimulate the production and distribution of these compounds in plants (Vacheron et al. 2013). Bacterial cytokinins extend the leaf's assimilation capacity, thereby increasing the amount of photosynthesis products and replacing plant cytokinins lost during drought conditions (Arkhipova et al. 2007).

Bacteria associated with disease resistance responses can also act as biocontrol factors. The reduced susceptibility to pathogens can be related to the role of competitive colonization of tissues, or with antibiosis, consisting of interrupting the metabolism or degrading pathogens or toxins of pathogens or virulence factors or by activating (priming) the resistance of plants (Compant et al 2013). Bacteria also protect plants before attack by predators.

According to Ludwig-Müller (2015), plants and endophytes are equal partners in the production of secondary metabolites and can interact in the production of compounds and produce new metabolites for both organisms. For example, Scherling et al. (2009) showed that in poplar tissue cultures inoculated with *Paenibacillus* sp., 11 metabolites were altered in the plant, mainly those related to nitrogen assimilation.

In contrast to many beneficial effects that can be attributed to the plant's microbiota, some microorganisms can also pose a danger to human health, as the plant provides a niche where, in addition to the host's indigenous microbiota, opportunistic human pathogens can also establish themselves (Rastogi et al., 2012). In addition to direct pathogenicity, bacteria associated with the plant can serve as carriers of antibiotic resistance and potentially spread antibiotic resistance genes to human pathogens (Cernava et al., 2019). Recent findings related to the plant's microbiome provide evidence that the plant's bacterial community is influenced by specific assembly factors, including soil type, plant compartment, host genotype/species, plant immune system and plant development stage (Berg et al., 2016; Karlsson et al., 2017).

4.3 Bacteria associate with *Camellia sinensis*

The healthy appearance of tea trees is an indication that the microbial taxon of the rhizosphere is playing a key role in suppressing the incidence of disease and increasing the nutrient cycling and healthy soil status (Bora, et al. 2021). Bacteria such as *Rhizobacteria, Azotobacter, Azospirillum, Bacillus amyloliquefaciens, Bacillus pumilus, Serratia marcescens* and *Pseudomonas* have already been tested for beneficial activities in tea plantations in Northeast India, showing that most of them exhibited promising characteristics for promoting plant growth, such as solubilization of minerals, production of siderophore and indole acetic acid and display of biocontrol activities (Nath et al. 2013). It was also shown that these bacteria contributed positively to the germination of tea seeds and seedling growth in a nursery and subsequent field establishment (Bhattacharyya and Sarmah 2018).

A recent work has evaluated potential N2-fixing bacteria, such as *Azotobacter* and *Azospirillum*, showing organic changes that played an important role in the nitrogen geocycle in tea soils (Gebrewold 2018). These bacteria derive nutrients from the root exudates and, in return, provide fixed nitrogen efficiently to the host plant (Tejera et al. 2005). In addition to fixing atmospheric nitrogen, *Azotobacter* also secretes important phytohormones (IAA, gibberellic acid

and cytokinins), vitamins (thiamine, riboavin) and various antipathogenic agents that promote growth, as well as suppress the incidence of disease (Sumbul et al. 2020).

Another study also showed the importance of *Azospirillum* in supplementing nutrients in seedlings and tea cuttings propagated vegetatively (Thomas et al. 2010). *Azospirillum*, once inoculated in the soil, multiplies and spreads rapidly in the microaerobic sites of the plant's roots, intercellular spaces and symbiotically assists the host plant in the absorption of nutrients (Fukami et al. 2018). *Azospirillum* also produces a series of bioactive compounds, such as phytohormones (IAA and GA), siderophores, poly β-hydroxybutyrate that can influence the architecture of the root through mineral nutrition, development of the root hair, etc. (Egamberdieva et al. 2017).

Bora, et al. (2021), evaluated the microbial population in *C. sinensis* plants and showed that the distribution at the class level, that the rhizosphere was highly populated by *Proteobacteria* (40.98%), *Acidobacteria* (19.74%), *Firmicutes* (14.43%), *Actinobacteria* (10.95%), *Bacteroidetes* (4.70%), *Verrucomicrobia* (3.05%) and *Cyanobacteria* (1.9%); while the endosphere was inhabited by *Proteobacteria* (89.17%), *Actinobacteria* (5.08%), *Bacteroidetes* (1.52%), *Firmicutes* and *Synergistetes* (both with 0.85%). Most of these bacteria have been reported to either promote plant growth or protect the plants from pests and diseases (Fernández-González et al. 2017; Verma et al. 2019).

Bora, et al (2021) also showed that the species *Azotobacter chroococcum, Bacillus circulans, Bradyrhizobium japonica, Pseudomonas chlororaphis* and *Pseudomonas putida* exhibited promising activities of phosphate solubilizing tea rhizobacteria. In a P deficient soil, phosphate solubilizing microorganisms can effectively modulate the root architecture by promoting the lateral root and the root capillary system (Péret et al. 2014). The phosphate solubilization capacity of rhizosphere microorganisms are considered one of the most important characteristics associated with plant phosphate nutrient cycling (Alori et al. 2017).

Previously, cultivation-dependent approaches revealed that *Bacillus subtilis* was more dominant in acidic soils under tea cultivation (Bhattacharyya and Sarmah 2018). The following year, using the independent cultivation technique, metagenomics, Cernava, et al (2019) showed that C. sinensis plants were colonized predominantly by *Proteobacteria* (46.9–56.1%) and *Bacteroidetes* (40.1– 53.0%).

4.4 Bacteria in plant tissue culture

In practice, the initial explants are only sterilized on the surface and, thus, live microorganisms internally are introduced into *in vitro* cultures. Cultured microorganisms, even those that are not pathogenic, can have harmful effects on cultures and cause a lack of multiplication or variation in experimental results (Tsao et al. 2000). If symptoms of bacteria colonizing plant tissues appear in a short time, contaminated explants should be removed immediately. Undetected bacteria can start to multiply after a long period in culture (Kałużna, et al. 2013).

For many years, the presence of bacteria in tissue culture was generally not disclosed in published manuscripts and was considered derogatory for a laboratory, as *in vitro* cultures should be kept under sterile conditions. However, over time, it has become clear that, despite the sterilization of the surface of the initial explants, cultures are not necessarily free of bacteria. The presence of some bacteria may be apparent in the initial stage, but at other times, contaminants may not be detected until the multiplication stage, in which case bacterial growth is very slow or temporarily delayed under plant culture conditions, they remain in a cryptic state and can appear only when the culture conditions change drastically, for example, when suboptimal conditions or acclimatization can stimulate the growth of the bacteria (Dunaeva and Osledkin 2015).

A frequently used method, in addition to external sterilization, is the addition of antibiotics to the culture medium to suppress bacterial growth (Bohra et al. 2013). Herman (1990) introduced the idea that bacteria that are generally non-pathogenic or even growth-promoting can become harmful under the special growth conditions of *in vitro* culture and proposed the term "vitropaths". In contrast, several studies indicate that endophytes have a positive effect on plants, for example, through the biosynthesis of growth-stimulating phytohormones, increased availability of nutrients and induced resistance to pathogens (Goh and Vallejos 2013).

4.5 *In vitro* application of bacteria

Numerous bacterial taxa were isolated from plant tissue cultures and some of them showed the possibility of increasing plant growth rates or influencing *in vitro* morphogenesis. Compared to the production of *ex vitro* plants, it is easier to adjust the ideal conditions that favor the growth and organogenesis of explants during *in vitro* cultivation, both for research and for industry (Orlikowska, et al. 2017). Genotypes that are recalcitrant or difficult to grow do not respond with

effective regeneration, a high multiplication coefficient or effective rooting. In this case, the inoculation of cultures with beneficial bacteria can help in overcoming recalcitrance, supporting cultures with growth regulators or other metabolites (Orlikowska, et al. 2017). For example, *Rhodobacter sphaeroides* produces the phytohormone rodestrine, increasing the rooting of mulberry cuttings (Sunayana et al. 2005) and *Bacillus* spp. IAA production promoted the rooting of the strawberry (Dias et al. 2009). Quambusch et al. (2014) found a link between endogenous bacteria and the effectiveness of micropropagation of *Prunus avium* genotypes.

The most important role of beneficial bacteria in the micropropagation industry is related to the acclimatization of microplants, which is often an obstacle to the process. Non-functional (open) stomata, non-fully functional roots and the photosynthetic apparatus make adaptation difficult for microplants in rapidly changing conditions. The inoculation of microplants in the last stage of propagation or in suspension with beneficial bacteria could help to overcome this challenge (Panigrahi et al. 2015).

According to Orlikowska, et al. 2017, bacteria belonging to 13 genera (*Azorhizobium, Azospirillum, Azotobacter, Bacillus, Burkholderia, Curtobacterium, Enterobacter, Halomonas, Methylobacterium, Microbacterium, Methilophylus, Paenibacillus, Pseudomonas, Ralstonia*), stimulated stem length, increased weight, stem leaf number, axillary stem growth, accelerated rooting, increased the rate of rooted branches, increased the number and length of roots, induced somatic embryogenesis and helped in acclimatizing plants in vitro to *ex vitro* conditions.

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CHAPTER II

Purification and structural identification of the biosurfactant produced by the yeast *Wickerhamomyces anomalus* CCMA 0358

1 INTRODUCTION

Biosurfactants are molecules of biological origin capable of reducing surface and interfacial tensions (Ribeiro, Guerra and Sarubbo, 2020). Biosurfactants are secondary metabolites produced by microorganisms, associated with growth during the stationary phase of using oil as a carbon source (Sharma and Pandey, 2020). The surfactant activity of these natural compounds is due to the existence of hydrophilic and hydrophobic fractions in the molecular structure (López-Prieto et al., 2019). It has been established that microorganisms apply this metabolite as a tool to emulsify heavy oil chains to make them more bioavailable for effective use during their growth in the medium (Akbari et al., 2018). However, commercially available synthetic surfactants are more favored, when compared to biosurfactants, due to their low-cost (Jin et al., 2019).

Nevertheless, biosurfactants have aroused considerable interest in recent decades due to their advantageous properties over synthetic surfactants, such as high selectivity and biodegradability, stability in a variety of environmental conditions (temperature, pH and salinity) and low toxicity, favoring their application in recovery hydrophobic compounds and as emulsifiers in the pharmaceutical, cosmetic and food industries (Garg and Chatterjee, 2018).

To make the production of biosurfactants economically viable, it is important to reduce the cost of fermentation media, which represent about 50% of the final cost of the product (Jimoh and Lin, 2020). To this end, means that incorporate agro-industrial, industrial and domestic waste have been proposed as substrates to improve the viability of large-scale production of biosurfactants and make these natural products more competitive (Lima et al., 2020; Fernandes, et al. 2020).

Thus, a potential alternative to increase the production of biosurfactant and reduce costs, would be the use of renewable raw materials (Chen et al. 2018) and the use of yeasts, since most have GRAS status (*generally recognized as safe*) and have a high conversion rate of substrate (Fontes et al. 2008). However, although the production of yeast biosurfactants is known, few studies have investigated the influence on the metabolism and gene expression of yeasts related to the production of biosurfactant.

The yeast *Wickerhamomyces anomalus* CCMA 0358 was previously selected as the best producer of biosurfactant among other evaluated yeasts (Souza, et al, 2017). After its selection, the yeast *W. anomalus* CCMA 0258 had its production of biosurfactant increased through the development of an optimized culture medium using response surface methodology (Souza, et al, 2018).

Based on the ecological characteristics of the biosurfactants and their properties with biotechnological applications, this study aimed to purify and identify the structure of the biosurfactant produced by the yeast *Wickerhamomyces anomalus* CCMA 0358, as well as to evaluate the yeast gene expression during its production, when in KWO.

2 MATERIALS AND METHODS

2.1 Culture Conditions

The yeast *W. anomalus* CCMA 0358, isolated from coffee processing, was obtained from the Agricultural Microbiology Culture Collection, CCMA (Department of Biology, Federal University of Lavras, Brazil). The strain was grown in YEPG medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, pH 6.5) at 28 °C and 200 rpm for 48 hours. *W. anomalus* CCMA 0358 was monitored to be inoculated into the bioreactor at an initial cell concentration of 10^7 cells/mL.

2.2 Biosurfactants Production

A 5 L bioreactor, equipped with agitation, temperature measurement and control, pH meter and air injection, was used. The experiments were carried out at 28 ºC using 2 L of previously optimised culture medium, with the modification of the carbon source to kitchen waste oil (KWO) (yeast extract – 4.64 g/L, ammonium sulphate – 4.22 g/L, glucose – 1.39 g/L and 10 g/L KWO (Souza, et al., 2018), with the air injected into the bottom of the bioreactor with a constant flow. The bioreactor was programmed to maintain agitation at 500 rpm at 28 °C for 12 hours to produce biosurfactant. Cell growth was monitored using 15 ml samples collected at zero time, and after 12 hours of fermentation (Souza et al., 2018), a Neubauer chamber (Marienfeld GmbH, Germany) was used for cell counting. Samples were collected for the analysis of gene expression, the inoculum (T0), after 6 h of fermentation (K1) and at the end of fermentation (K2), as a control, the same culture medium was used, but replacing the KWO with glucose and 2 samples were collected in the same way, C1 and C2, after 6 h and 12 h respectively.

2.3 Recovery of Biosurfactant

To obtain the cell-free supernatant, the medium was withdrawn from the bioreactor and centrifuged for 10 minutes at 15.000 rpm and 4 ºC. The biosurfactant produced was recovered from the supernatant by adsorption chromatography using a glass column (430 mL) filled with Amberlite XAD-2 polystyrene resin (Sigma-Aldrich, USA), as described by Gudiña et al. (2010). Subsequently, 250 mL of the supernatant, obtained at the end of the fermentation through the column, was passed until the surface tension of the effluent was equal to or greater than 50 mN/m. After, the column was washed with three volumes of demineralised water to remove the non-adsorbed compounds. The biosurfactant adsorbed on resin was eluted with 750 mL of methanol, then a rotary evaporator at 60 °C and 120 rpm was used to concentrate the material.

2.4 Characterization of the biosurfactant

2.4.1 Test for glycolipid confirmation

Methylene blue analysis procedure was performed according to Pinzon and Lu (2009), in which the pH of the sample was first adjusted to 2.3 ± 0.2 using 1N HCl. The acidified sample was extracted with five times the volume of chloroform. Four milliliters of the chloroform extract were carefully removed and placed in contact with a freshly prepared methylene blue solution, containing 200 μL of 1 g / L methylene blue reagent, and 4.9 mL of distilled water. The pH of this aqueous methylene blue solution was pre-adjusted to 8.6 ± 0.2 by the addition of 50 mM borax buffer (~ 15 μL). After being mixed vigorously for 4 min, the samples were left to stand for 15 min. The chloroform phase was transferred to a cuvette and the absorbance was measured at 738 nm with a spectrophotometer against a chloroform blank. The absorbance values were converted to glycolipid concentrations using an established calibration curve, according to Marcelino, et. al. (2017).

2.4.2 Thin layer chromatography (TLC)

Thin layer chromatography of the crude biosurfactant was performed in reverse phase on glass plates coated with C18 silica gel (MilliporeSigma ™) using acetonitrile/methanol (3:1, v/v) as the solvent system. The chromatograms were visualized in visible and UV light (254 and 365 nm) before treatment with P-anisaldehyde-sulfuric acid reagent (mixture of 0.5 ml of P-anisaldehyde, 0.5 ml of concentrated sulfuric acid and 9 ml of ethanol). From the Rf value, the compound was identified.

2.4.3 Medium Pressure Liquid Chromatography (MPLC) analysis

The analysis and isolation of crude biosurfactant mixtures were determined by medium performance liquid chromatography (MPLC) using an FP SELECT C18 40g column. Acetonitrile and methanol were used as eluents at a flow rate of 45 mL/min and the injection volume was 400mg in dry load mode.

2.4.4 Liquid Chromatography–Mass Spectrometry (LC-MS)

For LC-MS analysis, the sample was diluted in H_2O and injected onto a Phenomenex Kinetex C18 column (2.1mm X 100 mm). A standard reverse phase gradient was run over 12 minutes at a flow rate of 250 µl/min and the eluent was monitored for either positive or negative ions via separate LC runs by a Thermo Fisher Scientific Q-Exactive HF (Bremen, Germany) operated in profile mode. Source parameters were 4.5kV spray voltage, capillary temperature of 275°C and sheath gas setting of 15. Spectral data were acquired at a resolution setting of 60,000 FWHM with the lockmass feature which typically results in a mass accuracy < 2 ppm.

2.4.5 Structural analysis – *Nuclear magnetic resonance* (*NMR*)

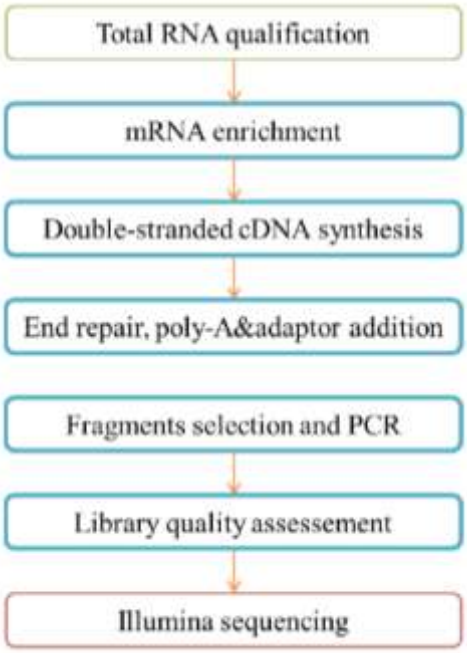
For NMR analysis, aliquots of the crude BS and of each fraction were dissolved in chloroform/methanol (3:1 v/v) and TMS as a standard in an NMR tube. The analyzes performed were ^{1}H, ^{13}C, DEPT 135, COSY, HSQC, HMBC and TOCSY.

2.5 Gene expression of yeast during the production of biosurfactant

2.5.1 Sample collection and preparation

From the RNA sample to the final data, each step, including sample test, library preparation, and sequencing, influences the quality of the data, and data quality directly impacts the analysis results. To guarantee the reliability of the data, quality control (QC) is performed at each step of the procedure (Figure 1).

Figure 1 – The workflow is as follows:



2.5.2 RNA quantification and qualification

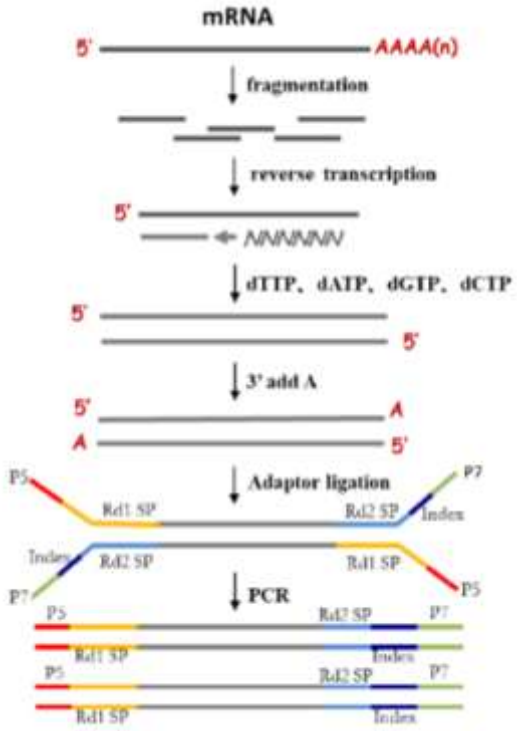
RNA degradation and contamination were monitored on 1% agarose gels. RNA purity was checked using the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). RNA integrity and quantitation were assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

2.5.3 Library preparation for transcriptome sequencing

A total amount of 1 μg RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using NEBNext® UltraTM RNA Library Prep Kit for Illumina® (NEB, USA) following manufacturer's recommendations and index codes were added to attribute sequences to each sample. Briefly as shown in Figure 2, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5X). First strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase (RNase H-). Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and Rnase H. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of 3' ends of DNA fragments, NEBNext Adaptor with hairpin loop structure were ligated to prepare for hybridization. To select cDNA fragments of preferentially 150~200 bp in length, the library fragments were purified with AMPure

XP system (Beckman Coulter, Beverly, USA). Then 3 µl USER Enzyme (NEB, USA) was used with size-selected, adaptorligated cDNA at 37 °C for 15 min followed by 5 min at 95 °C before PCR. Then PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. At last, PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system.

Figure 2 – RNA samples preparation.



2.5.4 Clustering and sequencing

The clustering of the index-coded samples was performed on a cBot Cluster Generation System using PE Cluster Kit cBot-HS (Illumina) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina platform and paired-end reads were generated.

2.5.5 Data Analysis

2.5.5.1 Quality control

Raw data (raw reads) of FASTQ format were firstly processed through fastp. In this step, clean data (clean reads) were obtained by removing reads containing adapter and poly-N sequences

and reads with low quality from raw data. At the same time, Q20, Q30 and GC content of the clean data were calculated. All the downstream analyses were based on the clean data with high quality.

2.5.5.2 Mapping to reference genome

Reference genome and gene model annotation files were downloaded from genome website browser (NCBI/UCSC/Ensembl) directly. Paired-end clean reads were mapped to the reference genome using HISAT2 software. HISAT2 uses a large set of small GFM indexes that collectively cover the whole genome. These small indexes (called local indexes), combined with several alignment strategies, enable rapid and accurate alignment of sequencing reads.

2.5.5.3 Novel gene prediction

Because transcriptome annotations are still incomplete, most RNA-seq studies will reveal novel genes and transcripts. The Stringtie was used to assemble the set of transcript isoforms of each bam file obtained in the mapping step. Gffcompare can compare Strintie assemblies to reference annotation files and help sort out new genes from known ones.

2.5.5.3 Quantification

Feature counts was used to count the read numbers mapped of each gene, including known and novel genes. And then RPKM of each gene was calculated based on the length of the gene and reads count mapped to this gene. RPKM, Reads Per Kilobase of exon model per Million mapped reads, considers the effect of sequencing depth and gene length for the reads count at the same time, and is currently the most used method for estimating gene expression levels.

2.5.5.4 Differential expression analysis

Differential expression analysis between two conditions/groups was performed using DESeq2 R package. DESeq2 provides statistical routines for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. The resulting P values were adjusted using the Benjamini and Hochberg's approach for controlling the False Discovery Rate (FDR). Genes with an adjusted P value < 0.05 found by DESeq2 were assigned as differentially expressed. (For edgeR without biological replicates) Prior to differential gene expression analysis, for each sequenced library, the read counts were adjusted by Trimmed Mean of Mvalues (TMM) through one scaling normalized factor. Differential expression analysis of two conditions was performed using the edgeR R package. The P values were adjusted using the Benjamini and Hochberg methods. Corrected pvalue of 0.005 and |log2 (Fold Change) | of 1 were set as the threshold for significantly differential expression.

2.5.5.5 GO enrichment analysis

Gene Ontology (GO) enrichment analysis of differentially expressed genes was implemented by the cluster Profiler R package, in which gene length bias was corrected. GO terms with corrected P value less than 0.05 were considered significantly enriched by differential expressed genes.

2.5.5.6 KEGG Pathway enrichment analysis

KEGG is a database resource for understanding high-level functions and utilities of the biological system, such as the cell, the organism and the ecosystem, from molecular level information, especially large-scale molecular datasets generated by genome sequencing and other high-through put experimental technologies (http://www.genome.jp/71eg/). Was used cluster Profiler R package to test the statistical enrichment of differential expression genes in KEGG pathways.

2.5.5.7 Alternative splicing analysis

Alternative splicing analysis was performed by the software rMATS, a statistical method for robust and flexible detection of differential AS from replicate RNA-Seq data. It identifies alternative splicing events corresponding to all major types of alternative splicing patterns and calculates the P value and FDR for differential splicing. These types include exon skipping (SE), alternative 5¢ splice sites (A5SS), alternative 3¢ splice sites (A3SS), mutually exclusive exons (MXE), and retained introns (RI).

2.5.5.8 Mutation analysis

Firstly, Picard tools and Samtools were used to sort, mark duplicated reads and reorder the bam alignment results of each sample. Then the tool HaplotypeCaller in GATK software was used to perform variant discovery. Raw VCF files were filtered with GATK standard filter method and other parameters (cluster: 3; WindowSize: 35; QD < 2.0 or FS > 30.0). Finally, ANNOVAR was used to functionally annotate genetic variants detected from diverse genomes with user-specified versions of genome builds.

2.6 Antibacterial assay

Antibacterial activity was performed by determining the Minimum Inhibitory Concentration (MIC) using the microplate dilution technique based on the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2012). Briefly, 100µL of sterile Tryptone Soy Broth (TSB) culture medium was dispensed in all wells and 100µL of crude biosurfactant, fraction

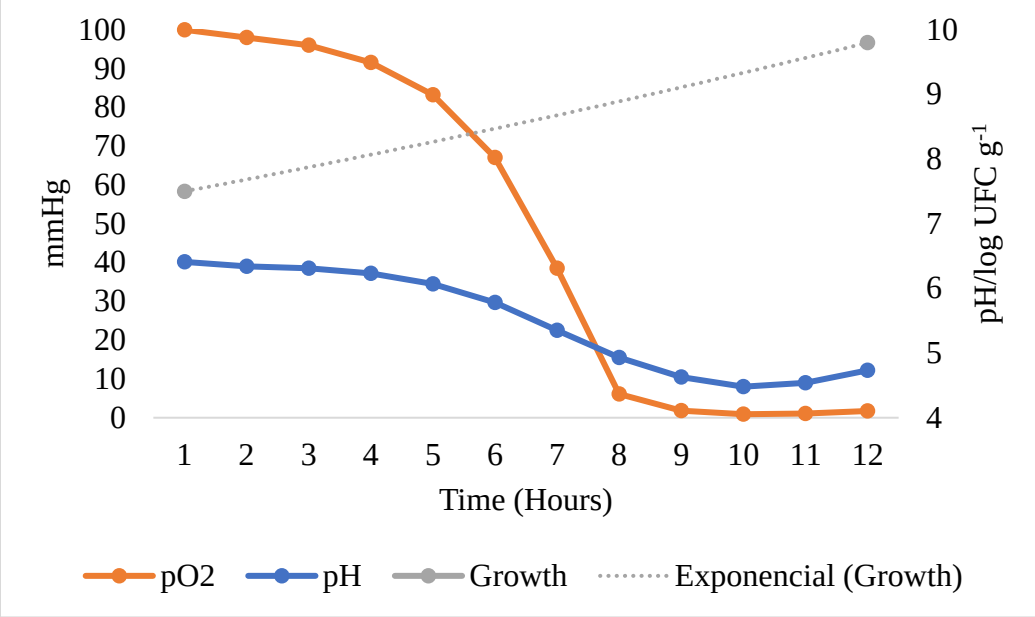
1 (NF1), fraction 2 (NF2) and co-fraction (NF1 + NF2) solutions (960 μg/mL) were added in the first column, serial dilutions were made to obtain final concentrations ranging from 3.75μg/mL to 480μg/mL. 10 μl of bacterial inoculum (*Bacillus cereus* ATCC 14579), at a concentration of 10^8 cells/mL, was transferred to all wells, except the negative control wells. The microplates were incubated at 28°C for 24h. MIC was determined to be the lowest concentration of biosurfactant that visually inhibited bacterial growth compared to the positive control (without biosurfactant). The Minimum Bactericidal Concentration (MBC) was determined by the microtip technique, spreading a sample from each well on TSB plates, subsequently incubated at 28°C for 24h. MBC was determined to be the lowest concentration where no viable growth was detected.

3 RESULTS AND DISCUSSION

3.1 Biosurfactant production

Figure 3 shows cell growth, O_2 and pH values during the 12 h fermentation to produce the biosurfactant by the yeast *W. anomalus*. Cell growth was assessed by counting in a Neubauer chamber, fermentation was started with 7.5 log UFC g^{-1} and at the end of the fermentation, after 12 h, a cell count of 9.8 log UFC g^{-1} was obtained. The air injected into the bottom of the bioreactor started with 100 mmHg and the consumption of O_2 by the yeast was measured, being 0.9 mmHg at the end of the fermentation. The pH was measured using a pH meter inserted into the bioreactor in contact with the culture medium, having an initial value of 6.41 and at the end of fermentation with a value of 4.54.

Figure 3 – Oxygen consumption by the yeast *W. anomalus*, evaluation of the pH values of the medium and yeast growth during the 12 hours of fermentation to produce biosurfactant.

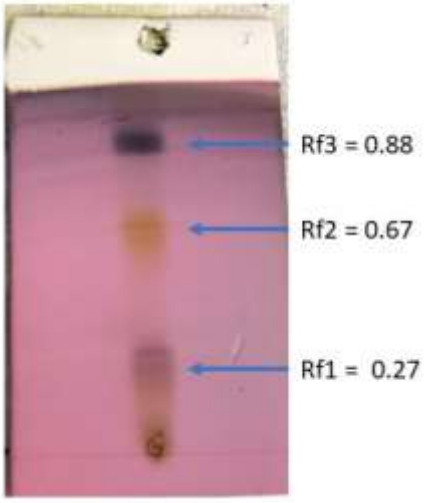


3.2 Characterization of the biosurfactant

In this study, the biosurfactant produced by *Wickerhamomyces anomalus* CCMA 0358 after 12 hours of cultivation, was characterized by TLC followed by MPLC for analysis and isolation of the crude biosurfactant mixture. The crude biosurfactant and the fractions obtained by MPLC were analyzed by LC-MS, MS, followed by structural characterization in NMR.

The reaction of the crude biosurfactant with the methylene blue in organic medium resulted in an absorbance of 0.545 (738 nm), indicating the presence of glycolipid biosurfactant. The blue color obtained in the reaction is due to the complexation between the glycolipid carboxyl groups and the methylene blue dye. This test was used in experiments with rhamnolipids produced by *Pseudomonas* and *Bacillus* (Pizon and Ju, 2009) and with glycolipid produced by yeast (Marcelino, et al., 2017). TLC analysis of the biosurfactant produced in the medium with KWO showed spots with retention factors (Rf) of 0.27, 0.67 and 0.88 (Figure 4), indicative of glycolipid production.

Figure 4 – TLC analysis of the biosurfactant produced by *W. anomalus* in KWO medium.

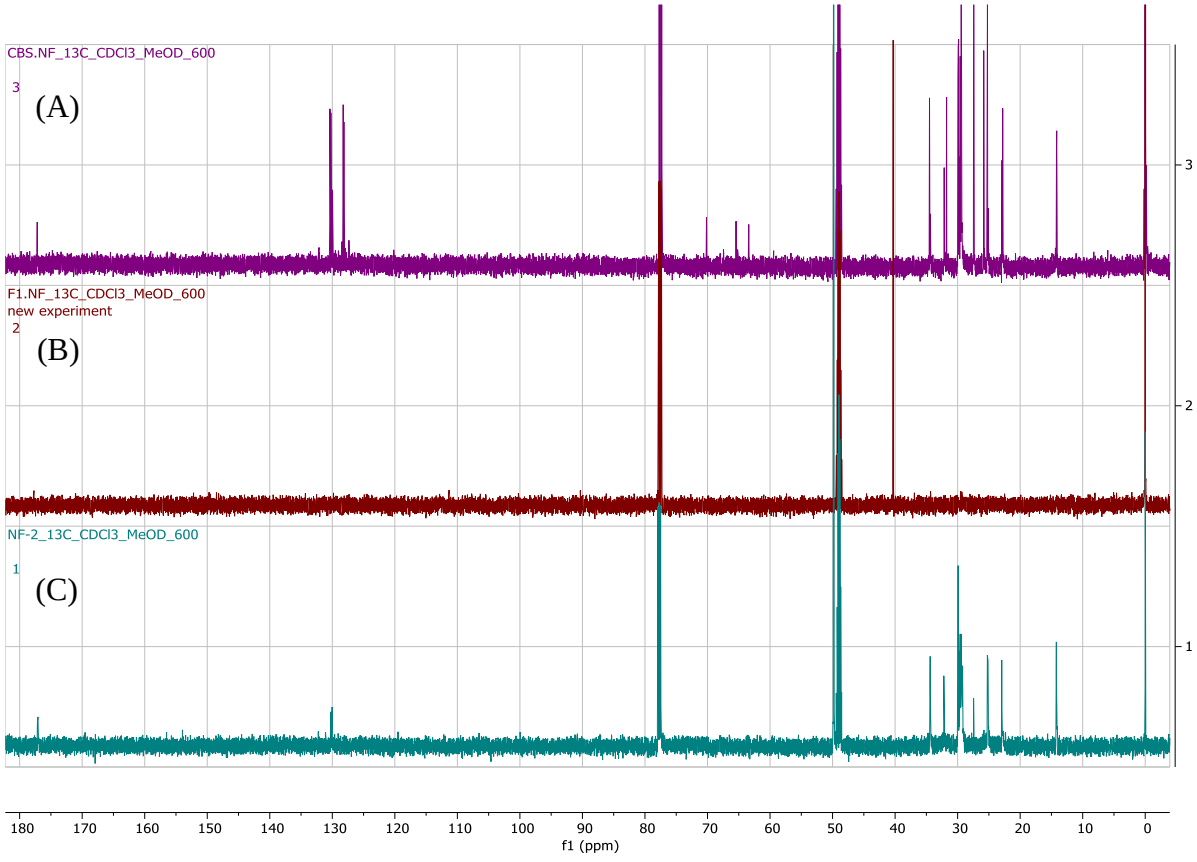


To better understand the structure of the biosurfactant molecule, the sample was purified by MPLC, resulting in two main fractions. These fractions and the crude bisurfactant were analyzed by ^{13}C NMR (Figure 5) and DEPT 135 (data not shown) and both spectra showed the number of carbons for the crude biosurfactant was 49, for the Fraction 1 was 4 and for the Fraction 2 was 43.

The spectrum of the crude biosurfactant in ^{13}C NMR (Figure 5.A) showed the presence of double bonds with signals at 130 ppm, showing at least 10 CH bonds. In addition, several CH_2 groups resonated at 22.79 - 34.35 ppm, showing at least 30 CH_2 bonds and two signals at 14.15 and 14.19 corresponding to two primary methyl groups (CH_3). The spectrum also revealed C-O bond signals at 63.4, 65.18 and 65.43 ppm, which could be from the glycerol chain. No signal was resonated close to 95 – 105 ppm (C1 sugar carbon). The ^{13}C NMR spectrum of the crude biosurfactant reveals at least three peaks at 177 ppm, typical of carbonyl groups. The presence of a glycolipid biosurfactant was expected, due to the glycolipid confirmation test. The result shows that the biosurfactant produced here, was not the same produced by Souza, et al (2017), in which the same strain of yeast was used, the same cultivation medium and the same conditions, being different only the carbon source offered to the yeast, so it was expected to also find a glycolipid biosurfactant. However, our spectrum shows that this biosurfactant does not have a sugar chain in its structure.

For Fraction 1 (Figure 5.B), the spectrum showed two peaks at 34.33 and 40.31, suggesting two CH_2 bonds, a peak at 95.86, suggesting a CH bond and a peak at 177.72, suggesting a carbonyl group. Fraction 1 did not have a good spectrum, suggesting that a more concentrated sample is needed, but its production was low, with Fraction 2 being the major compound in the crude biosurfactant mixture. The Fraction 2 spectrum at ^{13}C NMR (Figure 5.C) showed the presence of double bonds with signals at 130 ppm, showing at least two CH bonds. In addition, several CH_2 groups resonated at 22.95 - 34.46 ppm, showing at least 30 CH_2 bonds and a signal at 14.21 corresponding to a CH_3 group. The spectrum also revealed signs of an exchangeable halogen at 49.85 ppm, which could be the presence of chlorine or bromine in the structure. No signal was resonated close to 110 ppm (C1 sugar carbon). The ^{13}C NMR spectrum of Fraction 2 reveals at least three peaks at 177 ppm, suggesting the presence of carbonyl groups.

Figure 5 – ^{13}C NMR spectra obtained from the crude biosurfactant and its fractions produced by *Wickerhamomyces anomalus.*



Caption – A) ^{13}C NMR spectra obtained from the crude biosurfactant; B) ^{13}C NMR spectra obtained from the Fraction 1. C) ^{13}C NMR spectra obtained from the Fraction 2.

The chemical shifts of all protons and carbons couplings were determined from the HMBC spectrum and are assigned in Table 1. The HMBC spectra of all samples are shown in Figure 6 (Crude Biosurfactant), Figure 7 (Fraction 1) and Figure 8 (Fraction 2).

The HMBC spectrum of the crude biosurfactant (Figure 6) showed that 3 protons were coupled with 2 carbons, in the 0.88 ppm position with 22.77 and 31.8 ppm, in the 1.62 position with 29.31 and 34.3 and in the 2.06 ppm position with 29.48 and 31.44, being the carbons from the region known to be CH_2. The protons from the 2.05 and 2.77 ppm regions were also coupled with 2 carbons, but from the 130 ppm (CH) region, these 2 protons also share one of the carbons (128.11ppm).

For Fraction 1 (Figure 7), the HMBC spectrum showed that the carbon of the 40.15 ppm region was coupled with 3 protons (2.55, 2.66 and 2.78 ppm) and that the region of the 2.29 ppm region is coupled with the 24.88, 29.1 carbons (CH_2) and with the carbonyl group in the region 176.63 ppm. The HMBC spectrum of Fraction 2 (Figure 8) showed that the proton from the region 0.88 ppm is coupled with the carbons 22.87 and 31.99 ppm, carbons from the CH_2 region. Protons 1.61 and 2.99 are coupled with 2 carbons from the CH_2 region and one carbon from the carbonyl region.

Table 1 – HMBC NMR chemical shifts obtained from the crude biosurfactant and its purified fractions from the mixture.

HMBC - Chemical shift (ppm)					
	1H	**13C**		**1H**	**13C**
CBS	0.88	22.77	**Fraction 1**	0.89	23.09
	0.88	31.8		0.89	31.86
	1.26	29.63		2.29	24.88
	1.3	31.78		2.29	29.1
	1.33	29.31		2.29	176.63
	1.62	29.31		2.55	40.15
	1.62	34.3		2.66	40.15
	2.02	130.02		2.78	40.15
	2.05	128.11	**Fraction 2**	0.88	22.87
	2.05	130.11		0.88	31.99

2.06	29.48	1.26	29.57
2.06	31.44	1.29	32.38
2.28	177.07	1.33	28.89
2.29	25.12	1.61	29.18
2.29	29.14	1.61	34.23
2.29	24.86	1.61	176.88
2.34	29.08	2.01	29.57
2.35	174.51	2.01	130.27
2.77	128,11	2.29	24.81
2.77	130.02	2.29	29.18
5.34	27.4	2.29	177.17
		3.69	175.32
		5.25	27.19

Caption – CBS: Crude biosurfactant.

Figure 6 – HMBC NMR spectra obtained from the crude biosurfactant produced by *Wickerhamomyces anomalus.*

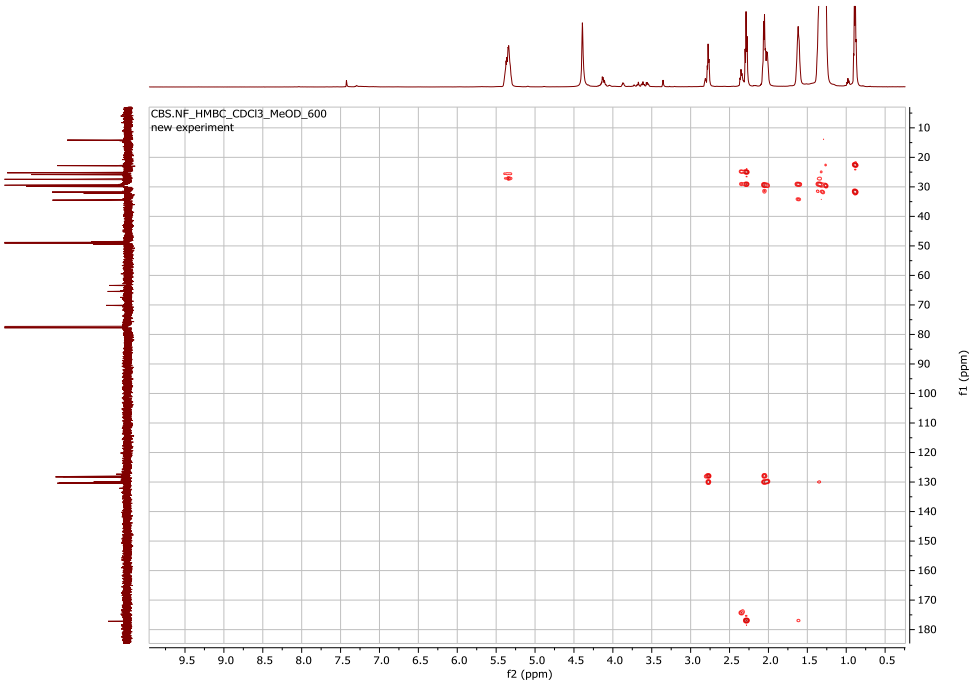


Figure 7 – HMBC NMR spectra obtained from the Fraction 1 obtained from the crude biosurfactant after MPLC.

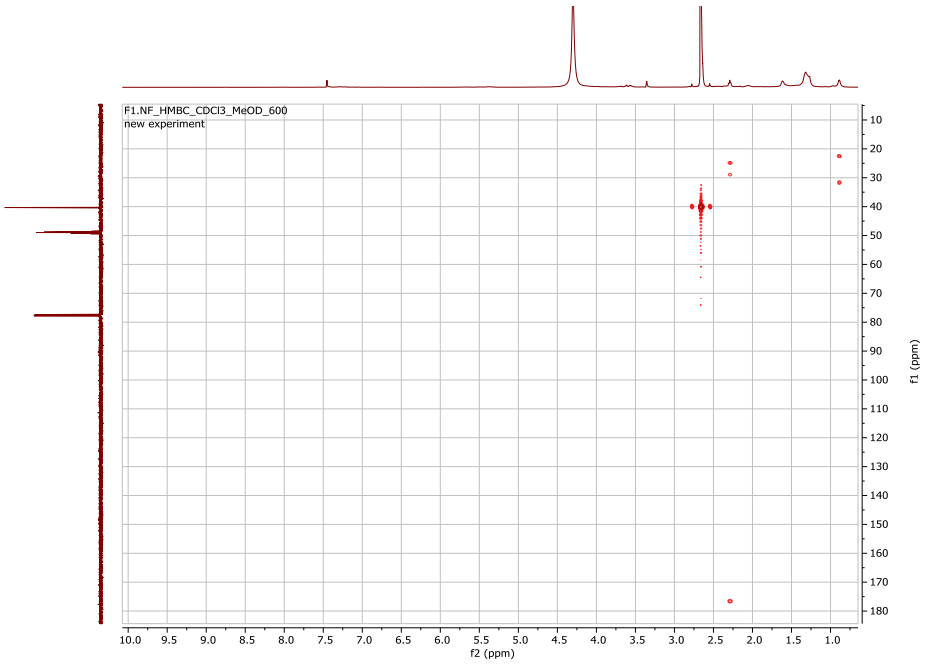
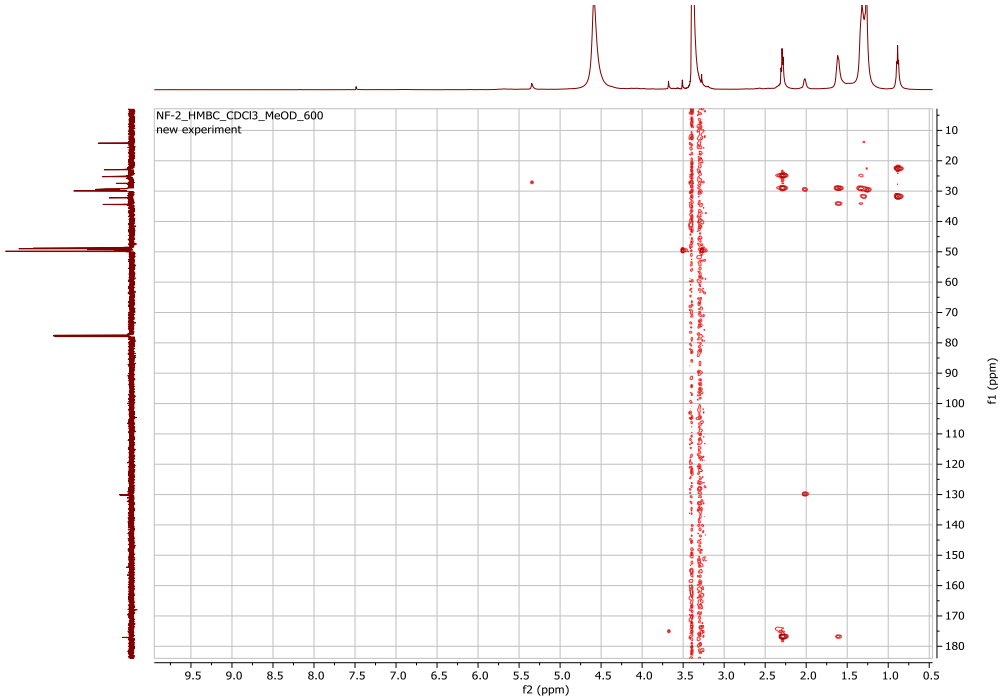
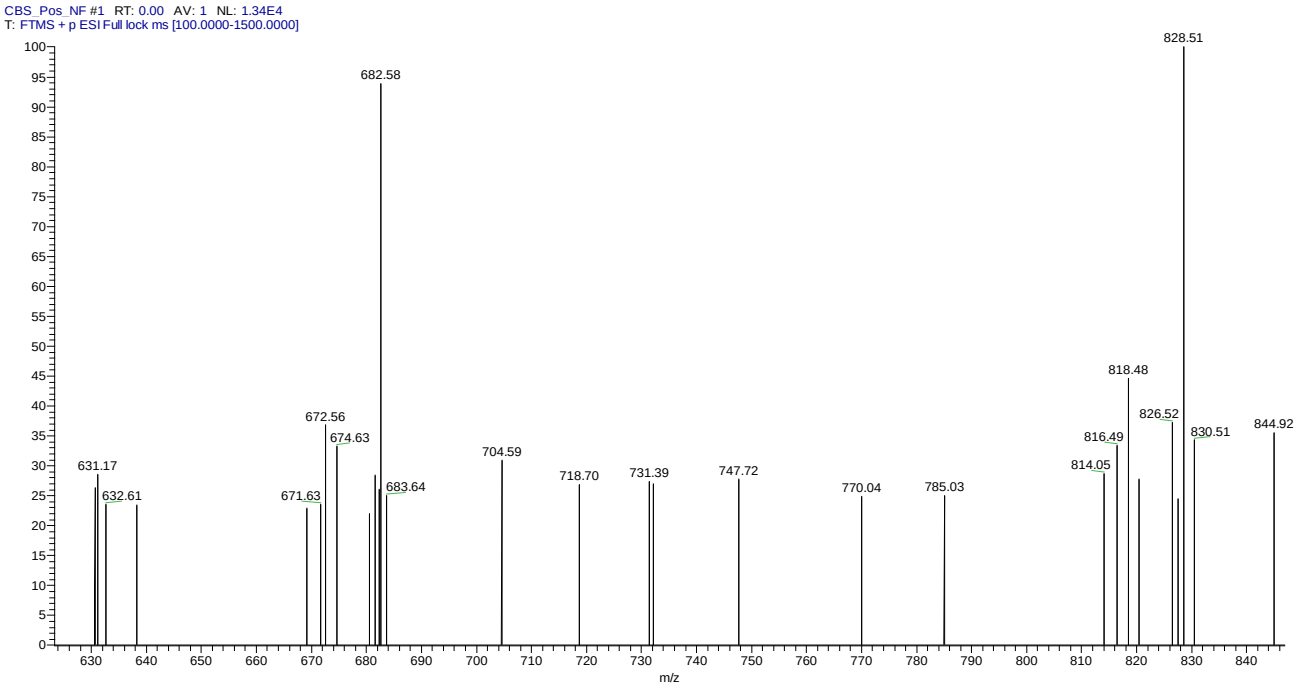


Figure 8 – HMBC NMR spectra obtained from the Fraction 2 obtained from the crude biosurfactant after MPLC.



The LC-MS analysis by direct infusion of the sample of crude biosurfactant dissolved in water (Figure 9), in the positive mode showed many peaks after 7 minutes of running (data not shown), showing itself in the MS spectra with major peaks of 682 and 828 m/z. According to the MS direct infusion, no important peaks were detected in LC-MS analysis in negative mode, which means that no important free acidic group is present in the biosurfactant molecule. After analyzing the masses, the spectrum was read by the Excalibur program and some structures were predicted. According to the data of ^{13}C NMR the structure of the crude biosurfactant would have around 49 carbons, with the presence of halogens, with that it was possible to taper the prediction. The predicted chemical formulas that most closely resemble were: $C_{48}H_{82}ONCl_4$; $C_{48}H_{79}O_5NBr$; $C_{49}H_{82}NCl_3K$.

Figure 9 – Positive mode mass spectrum of the crude biosurfactant.



According to the results obtained by Souza, et al, 2017, the MS analysis of the biosurfactant sample produced by *W. anomalus* CCMA 0358 using olive oil, showed a major peak at 903 m/z in positive mode and was classified as a glycolipid, being the lipophilic fraction composed of oleic acid. Two different types of glycolipidic biosurfactant structures were detected, containing one or three oleic acid molecules. However, our result shows that the biosurfactant produced here was not the same produced by Souza, et al (2017), in which the same yeast strain, the same cultivation

medium and the same conditions were used, however using KWO, therefore, it was also expected to find a glycolipid biosurfactant.

For Fraction 1, a major peak was shown at 871 m/z (Figure 10), however the ^{13}C NMR spectrum was not satisfactory to state the number of carbons present in the sample, so it was not possible to propose a chemical formula yet. Fraction 2 showed major peaks at 758 and 782 m/z in positive mode (Figure 11), also showing the characteristic isotopic peaks, which may be chlorine or bromine. Fraction 2 was given for having around 43 carbons, as a result, the Excalibur program was used for the prediction of chemical formula from the mass spectrum. The proposed chemical formulas were: $C_{43}H_{83}O_4NBr$; $C_{38}H_{79}O_9N_3Cl$; $C_{37}H_{73}O_4N_{10}Cl$; $C_{46}H_{83}OCl_3$.

Figure 10 – Fraction 1 positive mode mass spectrum.

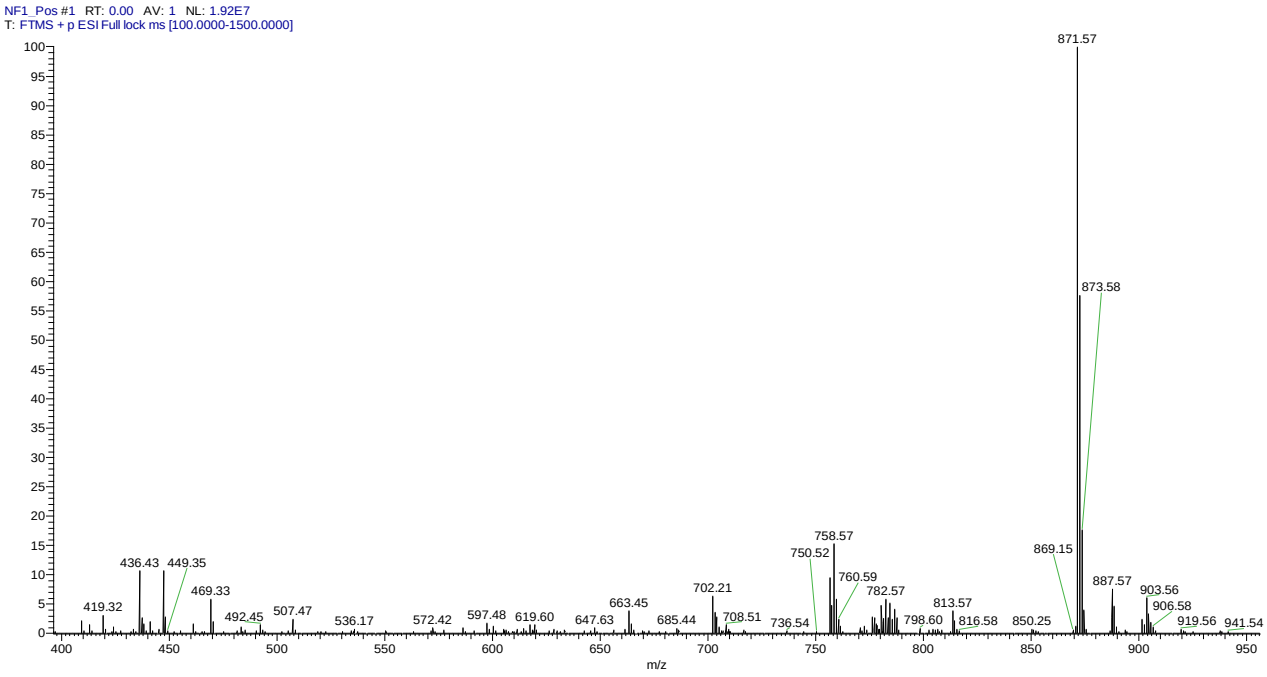
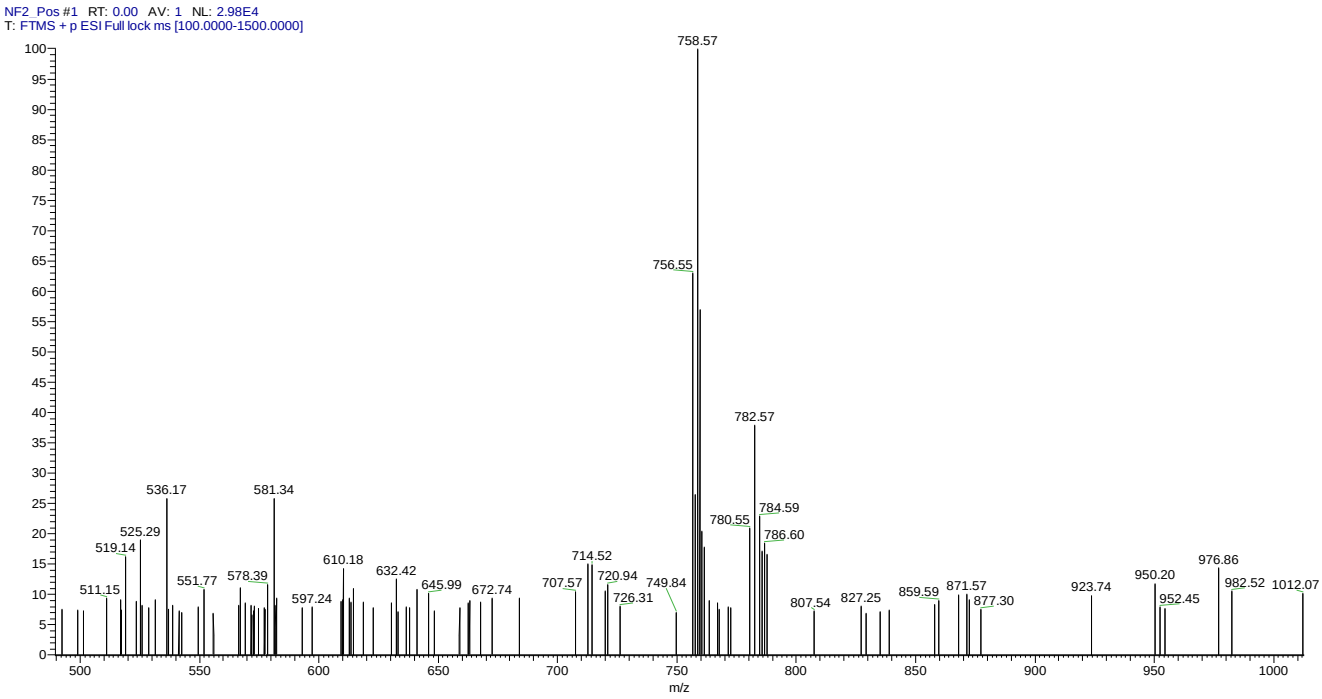


Figure 11 – Fraction 2 positive mode mass spectrum.



Pichia anomala PY1 has been previously reported to produce different sophorolipids in culture media containing glucose or soy oil as carbon sources, showing molecular weights of 659,499, 675,627 and 691,830 m/z, respectively (Thaniyavarn, et al, 2008). Therefore, the biosurfactant produced here is also different from that produced by *P. anomala*, as it had masses of 828, 871 and 758 m/z.

3.3 Gene expression of yeast during the production of biosurfactant

To detect all genes regulated by *W. anomalus* during the production of biosurfactant in KWO, RNA-Seq analysis of the complete transcriptomic profiles of the fermentation times and their control was performed. As shown in the Figure 12, a total of 240,816,958 clean readings were obtained from the five transcriptomes, and approximately 98% of these sequences were successfully matched to the reference *W. anomalus* genome, indicating the suitability of the sequencing data sets. Control (C), KWO (K) and samples with only inoculum (T) were harvested at different time points (0 = 0h; 1 = 6h; 2 = 12h).

Figure 12 – Statistics of the sequencing metrics generated from the RNA-Seq.

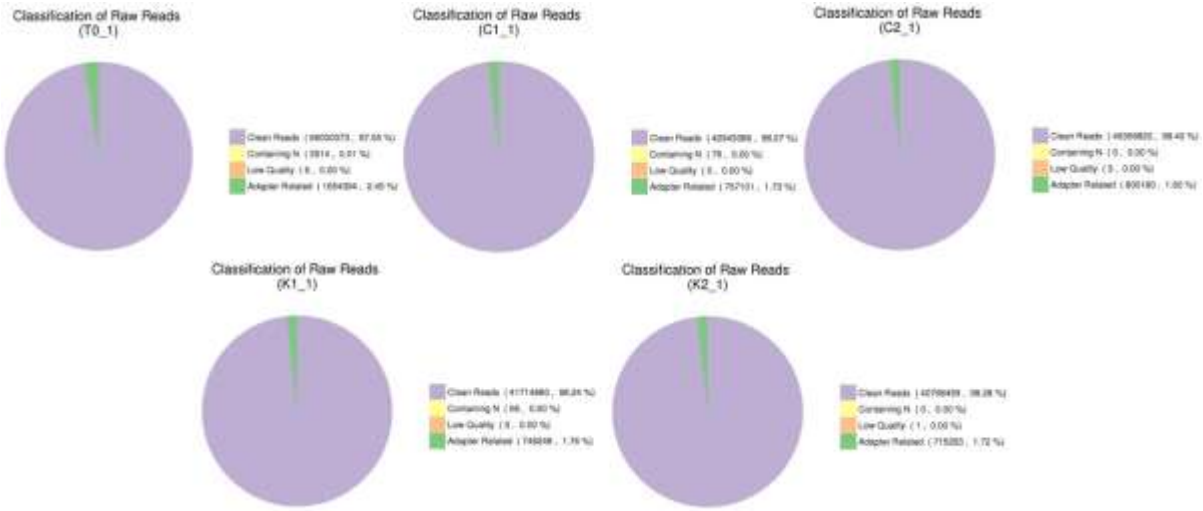
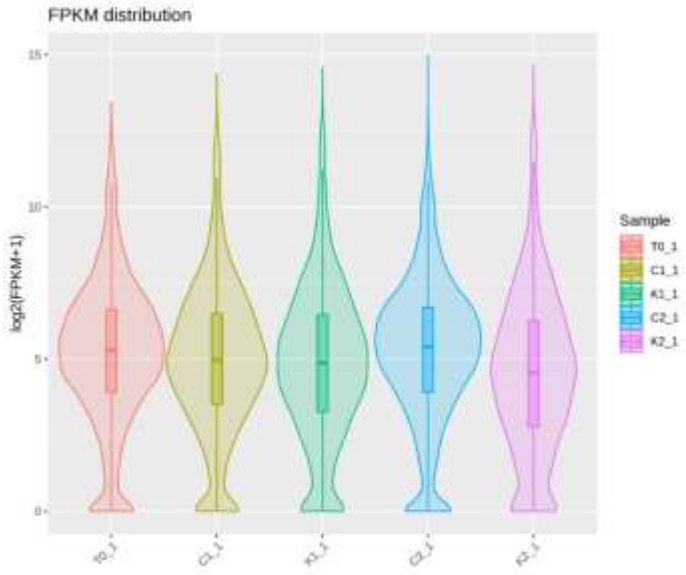


Figure 13 shows the fragments per kilobase of exon model per million reads mapped (FPKM) in the different treatments and sample times. It is possible to observe in the figure that the FPKM distribution in samples T0, C1 and C2 are greater than in samples K, as well as the transcription density of the expression. However, the distribution of FPKM is higher in samples C2 and K2, after 12h of fermentation.

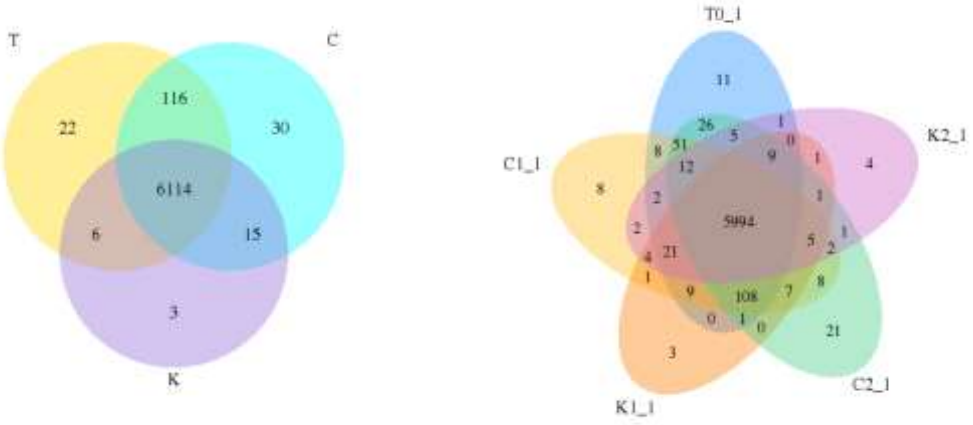
Figure 13 – FPKM distribution of transcripts.



Caption – The boxplot inside shows the FPKM distribution. The five characteristic values indicate maximum, upper quartile, average value, lower and minimum quartile, respectively. The violin diagram shows the density distribution of the FPKM. The width of the violin diagram represents the density of the transcription under a certain level of expression.

The Venn diagram shows differentially expressed genes (DEGs) between the samples (Figure 14). Diagram A shows that the 3 groups of samples share 6114 genes and that T expressed 22 specific genes, C expressed 30 and K expressed 3. The diagram also shows that T and C share 116 genes, T and K 6 genes and C and K 15 genes. Diagram B represents the sample DEGs, all of which share 5994 genes. Sample T0 expressed 11 specific genes, sample C1 expressed 8, sample C2 expressed 21, sample K1 expressed 3 and sample K2 expressed 4.

Figure 14 – Venn diagram of *W. anomalus* CCMA 0358 genes differentially expressed.

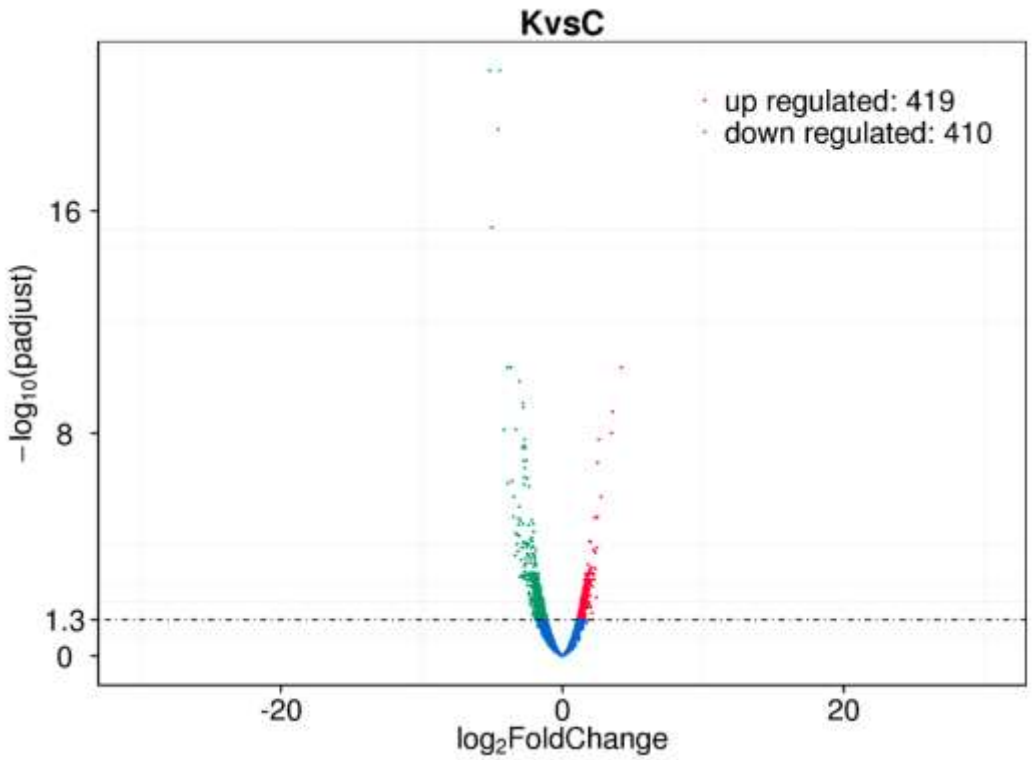


A) B)

Caption – In the Venn diagram, external numbers represent the total number of DEGs expressed specifically in each sample, while the numbers in the overlapping portions represent DEGs commonly expressed between samples. A) Groups of each treatment; B) Each sample of each treatment by sampling time.

Hundreds of genes were differentially expressed between 0 and 12 h of fermentation, in the treatment K (Figure 15). For the KWO sample (K), there were 829 differentially expressed genes in relation to the control (C). Among these genes, 419 DEGs were up regulated, that is, they were more expressed than in the control sample, and 410 DEGs were down regulated, i.e. less expressed than in the control.

Figure 15 – Volcano map of *W. anomalus* CCMA 0358 genes differentially expressed under treatments with KWO and control (without KWO).

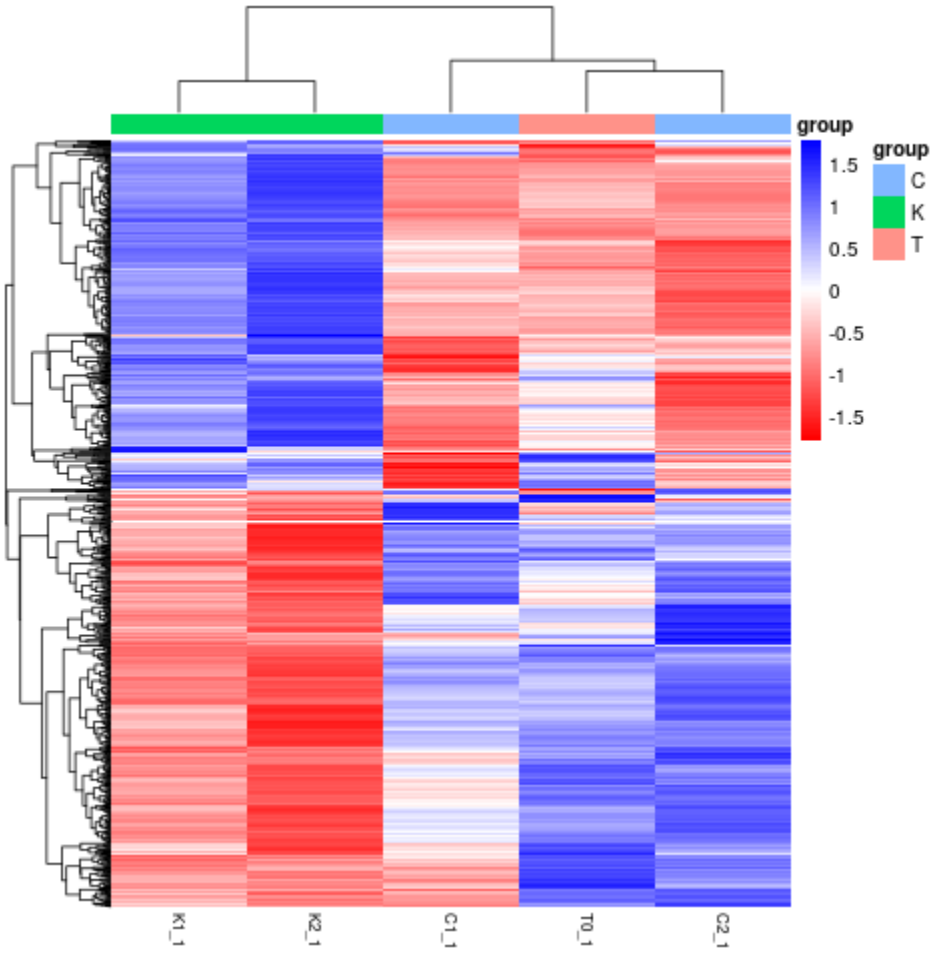


Caption – Differently expressed genes (DEGs). DEGs are shown as a red (up) or green (down) point. Blue dots represent genes that were not different between points in time. Abscissa represents several genes expressed in different samples. The values ordered on the axes represent the magnitude of the change in gene expression.

The heat map representation of the genes identified in the three groups of the yeast *W. anomalus* used during fermentation highlight distinct expression profiles among the samples (Figure 16). It is possible to observe in the figure that the two K samples, from the KWO fermentation, the genes were expressed in the opposite way to the C samples, control fermentation.

The first part of the genes expressed for K were up regulated and the second part of genes expressed was down regulated. As for samples C, the opposite happened, since in the first part, the genes were expressed down regulated and in the second part the genes were expressed up regulated.

Figure 16 – Heat map of expression levels of genes extracted during different times and treatments.



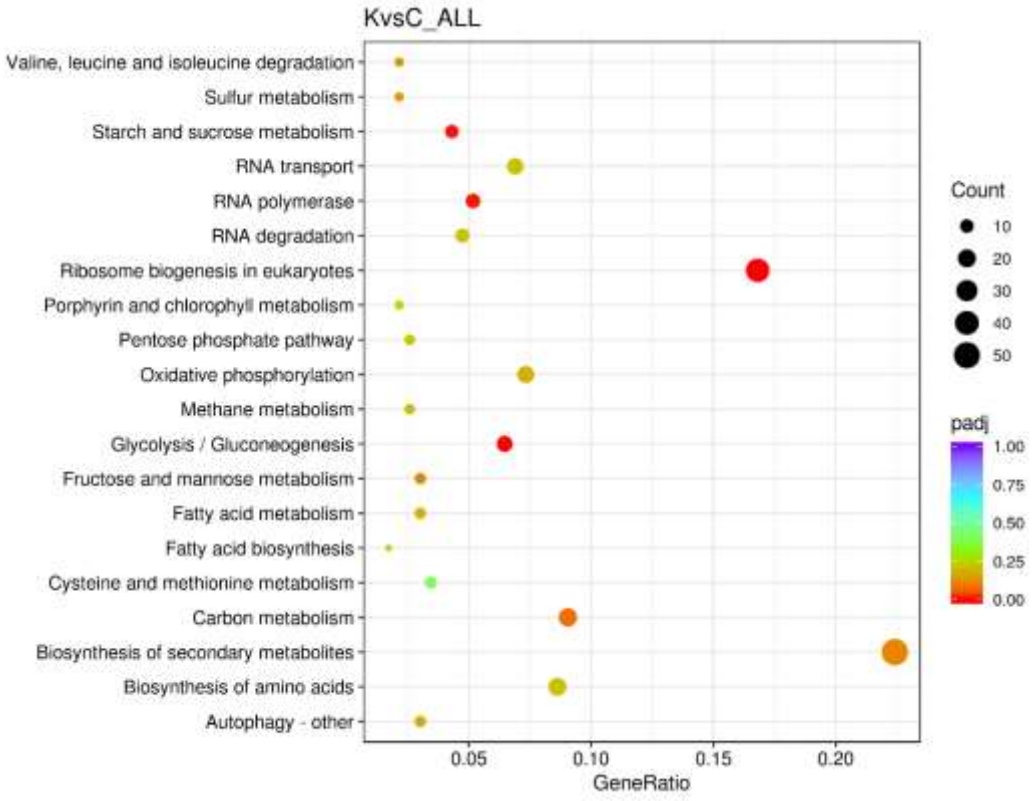
Caption – Blue = Up regulated. Red = Down regulated. Each row represents a gene, and each column represents a sample. The color and intensity of the boxes are used to represent changes (not absolute values) in gene expression.

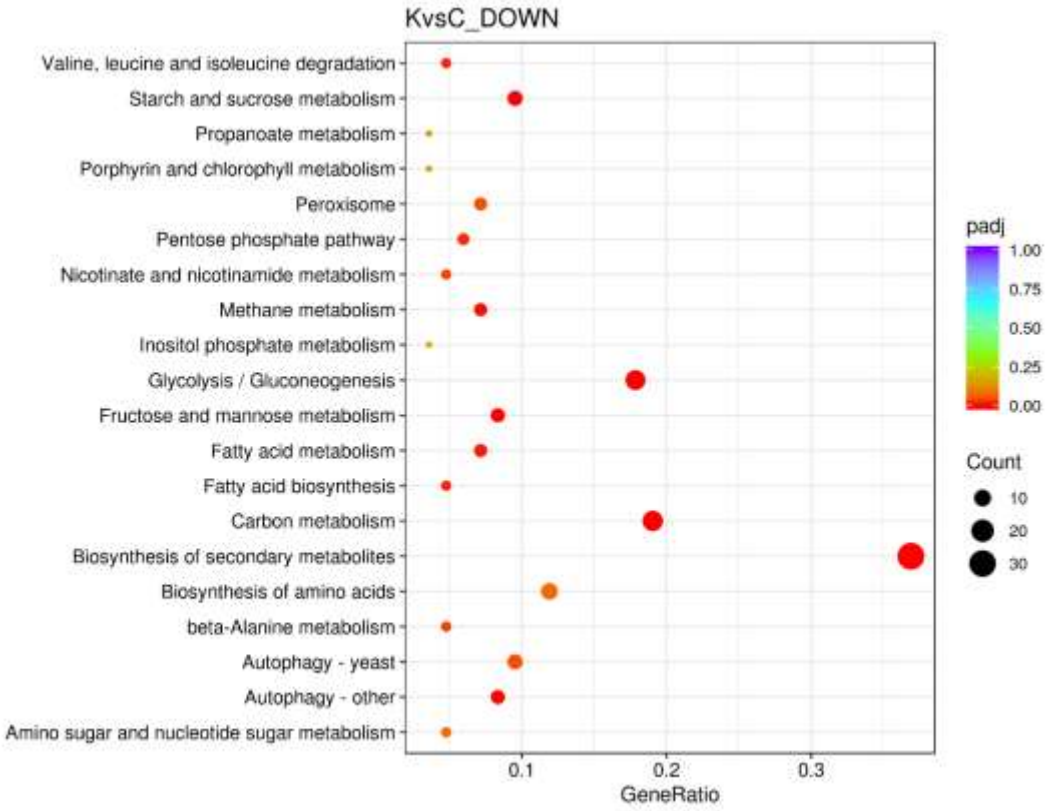
Based on gene expression data, the metabolic pathways in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database were regulated differently between groups C and K (Figure

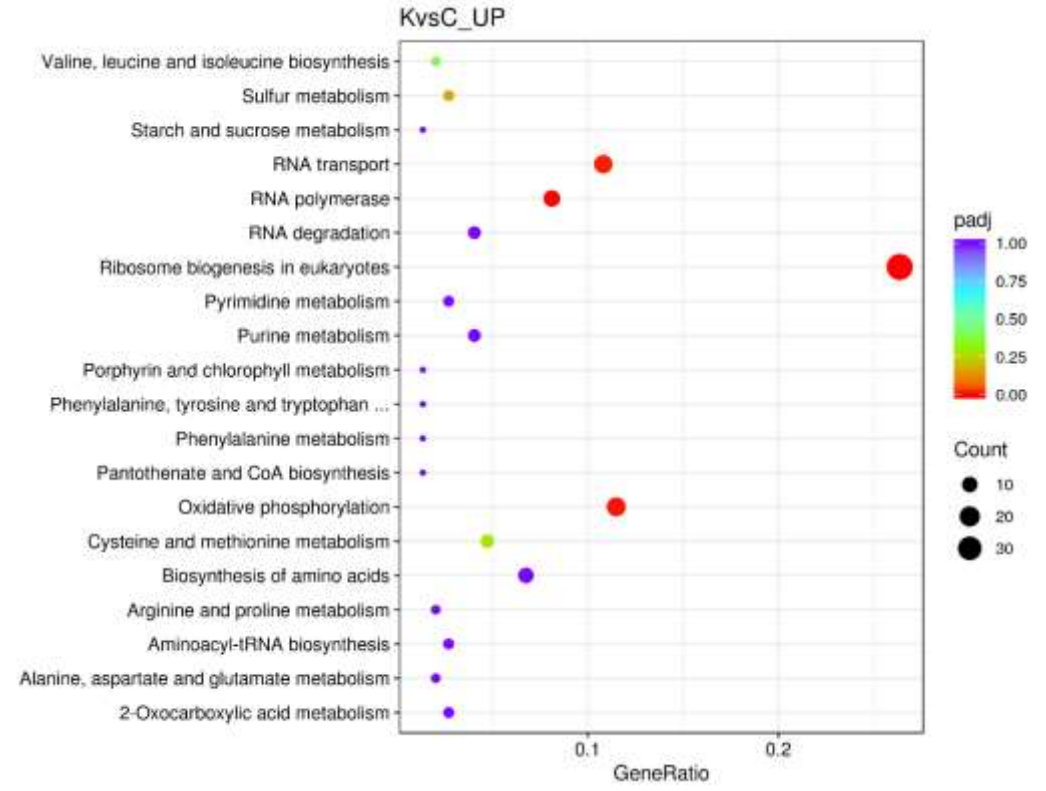
17). For all genes expressed (ALL), Ribosome biogenesis and biosynthesis of secondary metabolites were the two main enriched pathways. For genes less expressed in treatment K in relation to control (DOWN), Glycolysis / Gluconeogenesis, Carbon metabolism and Biosynthesis of secondary metabolism were the three main enriched pathways. Concerning the genes most expressed in treatment K in relation to control (UP), RNA transport, RNA polymerase, Ribosome biogenesis in eukaryotes and Oxidative phosphorylation were the 4 main enriched pathways.

Fatty acid biosynthesis for all genes expressed (ALL), Propanoate metabolism, Porphyrin and chlorophyll metabolism and Inositol phosphate metabolism for genes less expressed in sample K (DOWN) and Starch and sucrose metabolism, Porphyrin and chlorophyll metabolism, Phenylalanine, tyrosine and tryptophan, Phenylalanine metabolism and Pantothenate and CoA biosynthesis for the genes more expressed in the sample K (UP) were significantly low (Figure 12).

Figure 17 – The 20 most enriched (P value < 0.05) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways associate with *W. anomalus* CCMA 0358 during the fermentation to produce biosurfactant.




KvsC_DOWN
Valine, leucine and isoleucine degradation
Starch and sucrose metabolism
Propanoate metabolism
Porphyrin and chlorophyll metabolism
Peroxisome
Pentose phosphate pathway
Nicotinate and nicotinamide metabolism
Methane metabolism
Inositol phosphate metabolism
Glycolysis / Gluconeogenesis
Fructose and mannose metabolism
Fatty acid metabolism
Fatty acid biosynthesis
Carbon metabolism
Biosynthesis of secondary metabolites
Biosynthesis of amino acids
beta-Alanine metabolism
Autophagy - yeast
Autophagy - other
Amino sugar and nucleotide sugar metabolism
0.1
0.2
0.3
GeneRatio
padj
1.00
0.75
0.50
0.25
0.00
Count
10
20
30


KvsC_UP
Valine, leucine and isoleucine biosynthesis
Sulfur metabolism
Starch and sucrose metabolism
RNA transport
RNA polymerase
RNA degradation
Ribosome biogenesis in eukaryotes
Pyrimidine metabolism
Purine metabolism
Porphyrin and chlorophyll metabolism
Phenylalanine, tyrosine and tryptophan ...
Phenylalanine metabolism
Pantothenate and CoA biosynthesis
Oxidative phosphorylation
Cysteine and methionine metabolism
Biosynthesis of amino acids
Arginine and proline metabolism
Aminoacyl-tRNA biosynthesis
Alanine, aspartate and glutamate metabolism
2-Oxocarboxylic acid metabolism
0.1
0.2
GeneRatio
padj
1.00
0.75
0.50
0.25
0.00
Count
10
20
30

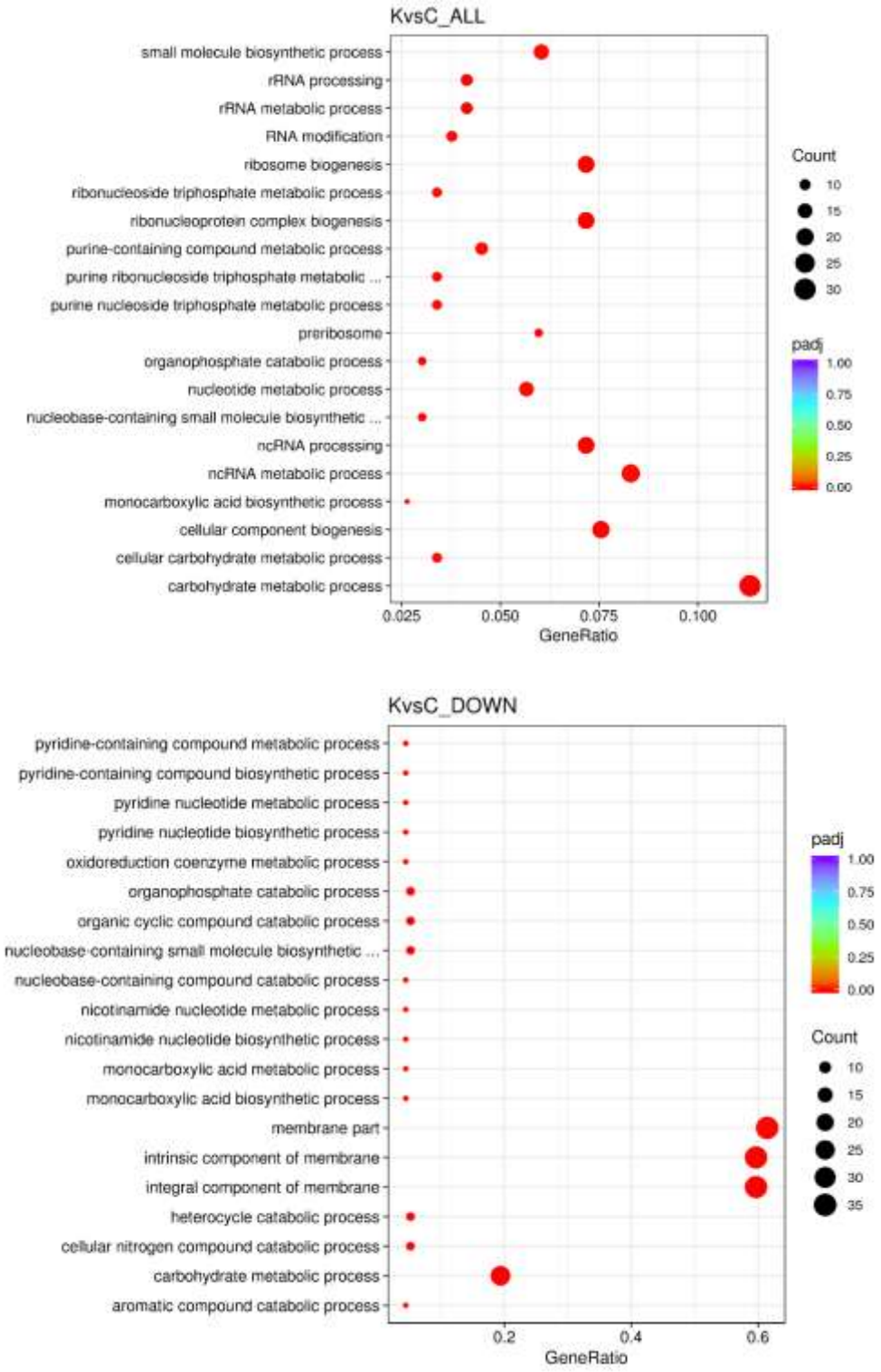
Caption – The Y axis represents the KEGG pathway, the rich factor of the X axis represents the percentage of candidate target genes enriched in the pathway, the padj value closer to 0 represents the most significant enrichment.

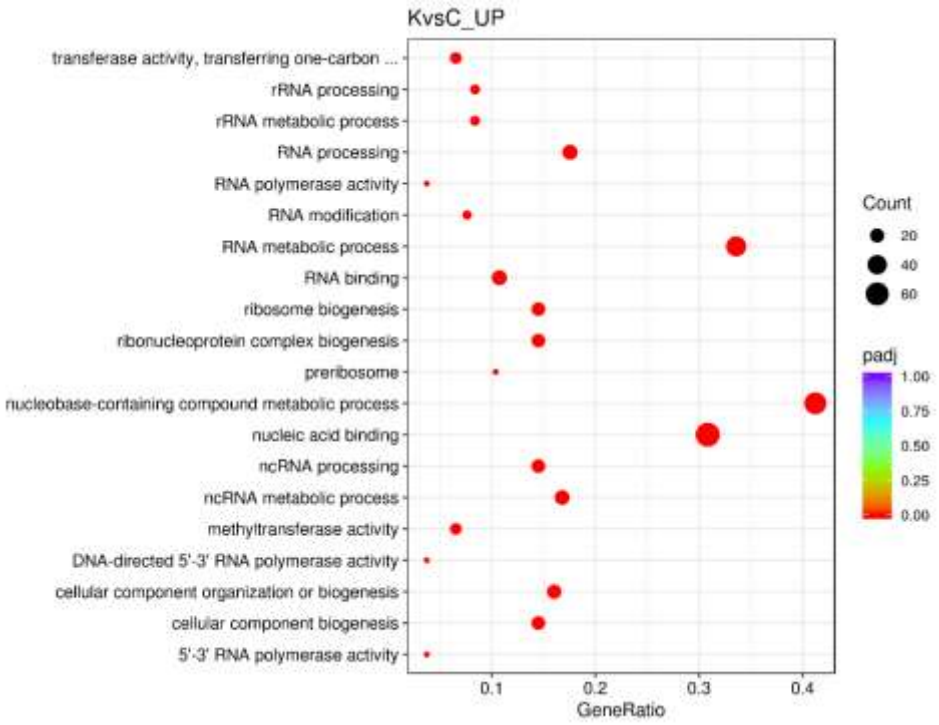
Kitchen waste oil contains a variety of residual vegetable oils, animal fats and salt, its high moisture and protein content causes its rapid degradation (Lan et al., 2015). This stimulates microbial growth and justifies the high oxygen consumption, shown in figure 3. Which can also be related to the increase in positively regulated genes expressed in K associated with oxidative phosphorylation, shown here in figure 17.

Rau, et al (2005) and Günther, et al (2015), showed that in the presence of vegetable oil, yeast cells expand and show a growing accumulation of globular inclusions that are characteristic of carbon storage. This cellular expansion is reflected by the transcriptomic profile in genes that encode enzymes and that are part of the machinery for cell growth, restructuring and modification of the cell wall and cell membrane, which may also justify the increase in K gene expression for growth functions, involving RNA (Figures 17 and 18).

Based on gene expression data, Gene Ontology (GO) annotates genes to biological processes, molecular functions, and cellular components that were regulated differently between groups C and K (Figure 18). For all expressed genes (ALL), ribosome biogenesis, ribonucleoprotein complex biogenesis, ncRNA metabolic process and carbohydrate metabolic process, were the main processes expressed. For genes less expressed in K than C (DOWN) the main components were related to the yeast cell membrane. For genes more expressed in K than C, the ones that stand out most are processes involving RNA.

Figure 18 – The 20 most enriched (P value < 0.05) Gene Ontology (GO) annotates genes to biological processes, molecular functions, and cellular components associate with *W. anomalus* CCMA 0358 during the fermentation to produce biosurfactant.





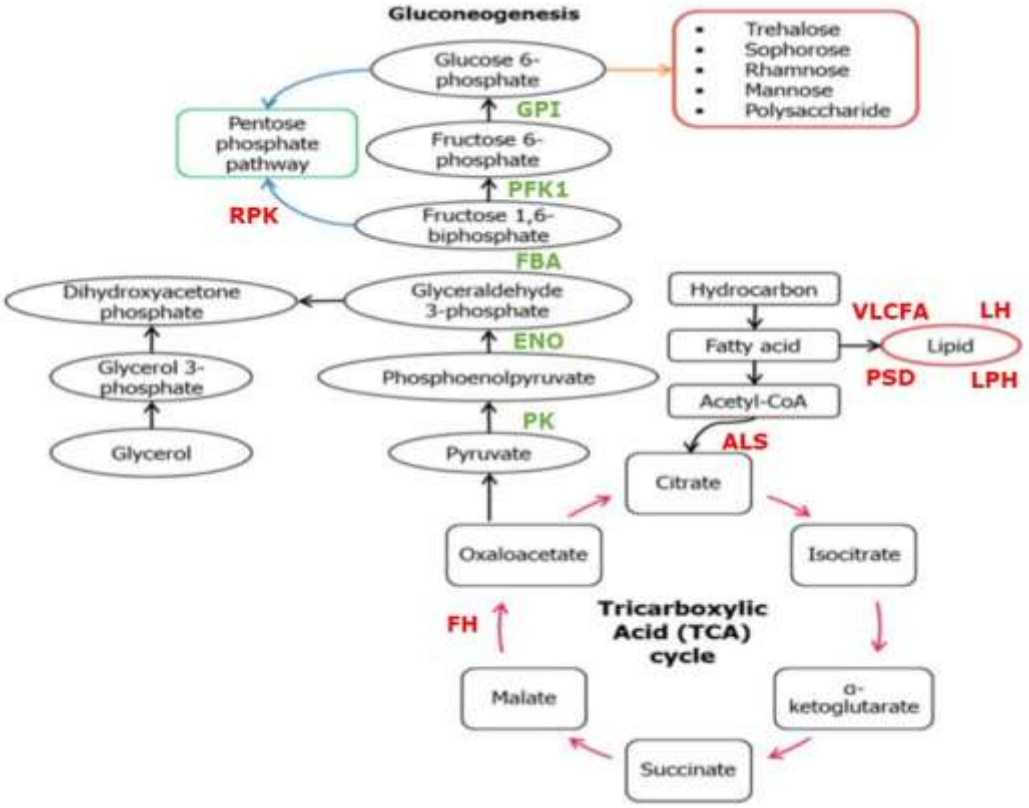
Caption – The Y axis represents the GO, the rich factor of the X axis represents the percentage of candidate target genes enriched in the pathway, the padj value closer to 0 represents the most significant enrichment.

According to Shekhar (2015), microorganisms that can grow on hydrophobic substrates, secrete large amounts of biosurfactant to facilitate the absorption of the carbon source. These microorganisms use a set of carbon and energy sources for growth. The combination of carbon sources with insoluble substrates facilitates intracellular diffusion, which may justify the genes that are being less expressed in K than in C related to membrane components, shown in figure 18.

Figure 19 shows the changes in gene expression in the main metabolic pathways, possibly involved in the biosynthesis of biosurfactants. The glycolytic enzymes phosphoglucose isomerase (GPI), 6-phosphofrutokinase 1 (PFK1), fructose 1,6-bisphosphate aldolase (FBA), enolase I (ENO), pyruvate kinase (PK) are being less expressed in K than in C, which reaffirms that, with a hydrocarbon as a substrate, yeast does not use glycolysis as the main ATP production pathway, concentrating its production on alternative pathways (Tokumoto, et al, 2009), such as the pentose phosphate pathway, which shows that ribose-phosphate pyrophosphokinase (RPK) enzyme is being more expressed, thus allowing the production of fatty acids or sugars, such as glucose, is

degraded until forming intermediates of the glycolytic pathway, such as glucose 6-phosphate, which is one of the main precursors of carbohydrates found in the hydrophilic moiety of a biosurfactant (Santos, et al, 2016).

Figure 19 – An overview of the expression changes of genes involved in the central metabolic pathways of yeast when in KWO to produce biosurfactant.



Caption – Genes upregulated are marked in red and genes downregulated are marked in green. The genes/enzymes that are not shown did not show differences in expression between the control and KWO.

Biosurfactants are partially derived from lipid metabolism that occurs in the cytosol and requires acetyl-CoA as a central precursor (Jezierska, et al, 2018). For its production to occur, the inactivation of the enzyme isocitrate dehydrogenase occurs, which leads to the accumulation of isocitrate and subsequently citrate in the mitochondria. Both acids are then transported to the cytosol, usually in exchange for malate, where the citrate is converted into oxaloacetate and acetyl-CoA (precursor to fatty acid synthesis) with the aid of acetolactate synthase I / III. This conversion

requires consumption of ATP and coenzyme A. The accumulation of citrate activates acetyl-CoA carboxylase, an enzyme that catalyzes the irreversible carboxylation of cytosolic acetyl-CoA into malonyl-CoA, the building block of new fatty acids. The newly formed fatty acid can then be directed towards lipid and biosurfactant biosynthesis (Albrecht, et al, 1996; Jezierska, et al, 2018).

Precursor genes for very-long-chain fatty acid (VLCFA) are being expressed more in K than in C, as shown in Figure 18, they are involved in lipid metabolism, fatty acid elongation, and biosynthesis of unsaturated fatty acids, which shows that VLCFA can be one of the precursors in the production of yeast biosurfactants. The enzymes lysophospholipid hydrolase (LH), phosphatidylserine decarboxylase of the mitochondrial inner membrane and serine esterase that deacylates exogenous lysophospholipids are also being more expressed in K than in C, being important enzymes in the metabolism of lipids and glycerophospholipids.

3.4 Antibacterial assay

The crude biosurfactant recovered from the fermentation of *W. anomalus* using KWO was evaluated and showed inhibitory (Figure 19) and bactericidal (Figure 20) activities against *Bacillus cereus*. The evaluated bacteria showed total inhibition at a concentration of 120 µg/mL and total bactericidal activity at a concentration of 480 µg/mL of crude biosurfactant. After the crude biosurfactant was purified, 2 fractions were given, then those fractions were analyzed against the same bacteria to determine whether the antibacterial activity would come from the set of fractions (crude biosurfactant) or if it would be more effective with the isolated fractions. Fractions 1 and 2 and the co-fraction showed inhibitory (Figure 21) and bactericidal (Figure 22) activities.

Fraction 1 showed the MIC of 240 µg/mL and the MBC of 240 µg/mL. Fraction 2 was shown to be the most effective, showing inhibitory and bactericidal activities against *B. cereus* in lower concentrations than crude biosurafactant and fraction 1, with 60 µg/mL MIC and MBC, proving that the isolated and purified compounds are more effective than the partially purified compound. Fractions 1 and 2 combined also showed inhibitory and bactericidal activities against the evaluated bacteria, with MIC and MBC of 60 µg/mL F1 + 60 µg/mL F2, which shows that fraction 2 is also more effective alone than combined with fraction 1.

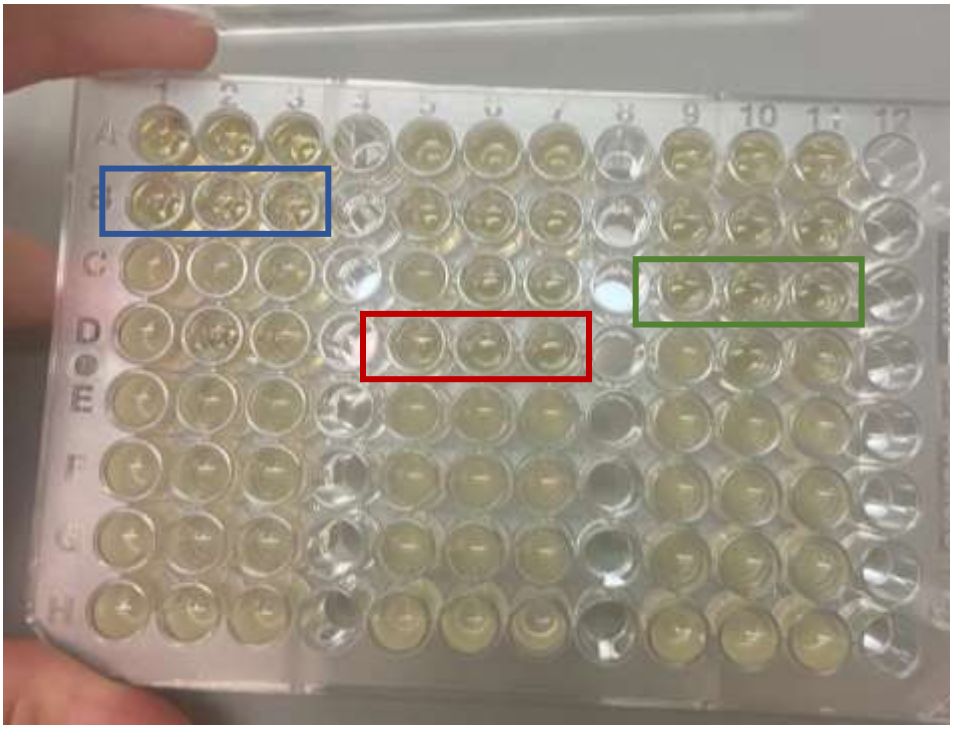
Figure 19 – Minimum inhibitory concentration of the crude biosurfactant of *W. anomalus*.

Caption – Concentrations: A – 480 µg/mL, B – 240 µg/mL, C – 120 µg/mL, D – 60 µg/mL, E – 30 µg/mL, F – 15 µg/mL, G – 7.5 µg/mL, 3.75 µg/mL. Red square: MIC.

Figure 20 – Minimal bactericidal concentration of the crude biosurfactant of *W. anomalus*.

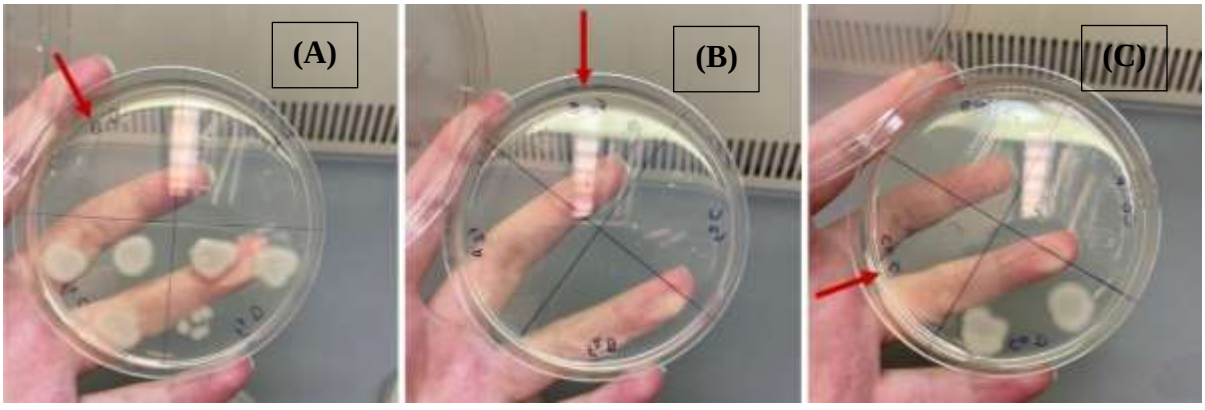
Caption – Concentrations: A – 480 µg/mL, B – 240 µg/mL, C – 120 µg/mL, D – 60 µg/mL, E – 30 µg/mL, F – 15 µg/mL, G – 7.5 µg/mL, 3.75 µg/mL. Red circle: MBC.

Figure 21 – Minimal inhibitory concentration of isolated and purified fractions 1 and 2.



Caption – Concentrations F1 e F2: A – 480 µg/mL, B – 240 µg/mL, C – 120 µg/mL, D – 60 µg/mL, E – 30 µg/mL, F – 15 µg/mL, G – 7.5 µg/mL, H – 3.75 µg/mL. Blue square: F1 MIC, red square: F2 MIC. Concentrations co-fractions F1+F2: A – 240 µg/mL F1 + 240 µg/mL F2, B – 120 µg/mL F1 + 120 µg/mL F2, C – 60 µg/mL F1 + 60 µg/mL F2, D – 30 µg/mL F1 + 30 µg/mL F2, E – 15 µg/mL F1 + 15 µg/mL F2, F – 7.5 µg/mL F1 + 7.5 µg/mL F2, G – 3.75 µg/mL F1 + 3.75 µg/mL F2, H – 1.87 µg/mL F1 + 1.87 µg/mL F2. Green square: Co-fractions MIC.

Figure 22 – Minimal bactericidal concentration of isolated and purified fractions 1 and 2.



Caption – A) F1 MBC, B) F2 MBC, C) Co-fractions MBC. Concentrations F1 e F2: A – 480 µg/mL, B – 240 µg/mL, C – 120 µg/mL, D – 60 µg/mL, E – 30 µg/mL, F – 15 µg/mL, G – 7.5 µg/mL, H – 3.75 µg/mL. Concentrations co-fractions F1+F2: A – 240 µg/mL F1 + 240 µg/mL F2, B – 120 µg/mL F1 + 120 µg/mL F2, C – 60 µg/mL F1 + 60 µg/mL F2, D – 30 µg/mL F1 + 30 µg/mL F2, E – 15 µg/mL F1 + 15 µg/mL F2, F – 7.5 µg/mL F1 + 7.5 µg/mL F2, G – 3.75 µg/mL F1 + 3.75 µg/mL F2, H – 1.87 µg/mL F1 + 1.87 µg/mL F2.

Recently, the Cybersan biosurfactant, isolated from the yeast *Cyberlindnera saturnus*, showed total inhibitory activity against *B. cereus* at a concentration of 200 µg/mL, MBC has not been demonstrated (Balan, et al, 2019), proving to be less effective than the produced biosurfactant in this study. Another study also showed the antimicrobial activity of a biosurfactant, produced by yeast of the genus *Candida*, against *Bacillus*, showing a MIC of 2000 µg/mL (Archana, et al, 2019), this study also did not show MBC results and was less effective than the antimicrobial activity presented here.

Biosurfactants, being amphiphilic molecules, help to reduce the hydrophobicity of the bacterial cell wall, being more potent in the Gram-positive bacterial membrane than in the Gram-negative bacterial membrane (Diaz de Rienzo, et al, 2016). Biosurfactants are generally known to inhibit bacterial growth, because they increase membrane permeability, followed by cytoplasmic leakage due to the formation of channels that still lead to cell death (Gaur et al. 2019). To emphasize the antimicrobial activity of the crude biosurfactant and its fractions produced in this study, more studies need to be done, evaluating its effects against more bacteria, gram negative and gram positive.

4 CONCLUSIONS

Biosurfactants produced by yeasts, using carbon sources that are renewable and cheaper have been studied over the years, but it is not fully understood which genes are involved in the genetic expression of yeast during the production of biosurfactants. In the present study, more than 6000 genes involved in its production were found, with 829 genes being differentially expressed between the control samples (without oil) and the treatment (using KWO). During fermentation with KWO the number of genes involved with functions related to RNA metabolism and transport, ribosome biogenesis and oxidative phosphorylation were more expressed, showing that the presence of oil in the culture medium increases the growth and multiplication of yeast, leading to

a greater production of biosurfactant. The use of KWO as a carbon source to produce biosurfactants clearly changes the profile of yeast gene expression, but more studies need to be done to punctuate these changes. The precursor enzymes for the production of biosurfactants were shown in this study, having a greater expression in precursors of the metabolism pathways of lipids and fatty acids, where one of the fractions of biosurfactants is produced. The crude biosurfactant and its isolated and purified fractions (F1 and F2) were also evaluated against the gram-positive bacteria *Bacillus cereus* and showed good antibacterial activity with MIC of 120, 240 and 60 µg/mL respectively and MBC of 480, 240 and 60 µg/mL respectively.

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CHAPTER III

Biosurfactants produced by yeasts: applications, screening, production, recovery, purification and characterization

OUTLINE:

1 Introduction – Provide a general introduction to what are biosurfactants (BS), their chemical composition, characteristics and properties.

2 Biosurfactants produced by yeasts – The most diverse advantages in the use of BS produced by yeast, due to its high rate of substrate conversion when compared with BS of bacteria, as well as the possibilities of greater application in industries.

3 Aplications of BS produced by yeast – In this section, several properties and industrial applications of BS produced by yeast are mentioned.

> **3.1 Medicine and Health –** Here we discuss the versatility and diverse properties of the BS produced by yeast that can be widely used in the pharmaceutical, medical and cosmetic industries.
>
> **3.2 Agriculture –** BS of yeasts that can be used as a complement to agribusiness, as an important auxiliary in products for biocontrol.
>
> **3.3 Bioremediation and Oil Recovery –** In this topic is discussed applications of yeast BS in bioremediation and how they stimulate the growth of oil-degrading microorganisms and improve their ability to utilize hydrocarbons.

4 Screening methods for detection of biosurfactants – Main methods used to screen, detect or evaluate potential BS-producing microorganisms and its advantages and disadvantages of each technique.

> **4.1 Hemolytic activity –** Here is shown the qualitative technique for screening BS-producing microorganisms using blood agar.
>
> **4.2 Blue agar plate method –** We show the technique developed for the detection of glycolipids that uses the culture medium with methylene blue and detects anionic BS by the formation of a dark blue halo around the culture.

4.3 Agar plate overlaid with hydrocarbons – In this topic, we show that this technique uses Luria-Bertani agar plates coated with hydrocarbons and, after the incubation period, colonies surrounded by an emulsified halo are detected as producing BS.

4.4 Axisymetric drop shape analysis (ADSA) – Here, we show the technique that determines the surface tension of the liquid from the profile of a drop and only the suspensions that produce BS show a reduction in surface tension.

4.5 Modified drop collapse method – It is shown the technique that uses a drop of the sample under a drop of oil, when the oil collapses is an indication of the presence of BS in the broth.

4.6 Oil spreading method – This technique uses oil over water and then the sample is added, if an emulsion halo is formed it is an indicator of the presence of BS.

4.7 Emulsification assay (EA) – This topic shows the method that uses absorbance after centrifuging the fermentation supernatant with a hydrocarbon to determine the EA.

4.8 Emulsification index (EI) – Here we show that the emulsion activity is measured by calculating the height of the emulsion for determining EI.

4.9 Tensiometric measurement – It is shown the measurement of surface tension using a tensiometer, in which cell-free supernatant is used.

5 Recovery and purification of biosurfactants – The latest advances in economic and efficient techniques to recover and purify biosurfactants from the fermentation broth.

5. 1 Acetone precipitation – After fermentation, the cell-free supernatant is mixed with ice-cold acetone to precipitate the emulsifiers.

5.2 Ethanol precipitation – This technique uses cell-free fermentation broth with ice-cold ethanol to precipitate the emulsifiers.

5.3 Ammonium sulfate precipitation – This method uses $(NH_4)_2SO_4$ for precipitation and is widely used in high molecular weight BS.

5.4 Acid precipitation – In this topic we discuss this method that is widely used in the recovery of BS because it is an easy, inexpensive and readily available method.

5.5 Centrifugation – It is shown that after acid precipitation, the broth containing BS can also be centrifuged to be easily collected as a crude product.

5.6 Crystallization – After the precipitation, the BS it is redissolved in an organic solvent, which crystallizes it.

5.7 Adsorption-desorption – In this topic is discussed the interaction of BS molecules with polystyrene resins and how it is used for the purification of BS.

5.8 Foam fractionation – This technique uses the basic principle of the separation of adsorptive bubbles and it is discussed in this topic.

5.9 - Solvent extraction – Here we shown this technique that uses the BS concentrated from the supernatant by the addition of $ZnCl_2$ and it is extracted twice with solvents.

6 Characterization of biosurfactants – Analytical chemistry techniques to prove the production and characterize surfactant compounds.

6.1 Thin layer chromatography (TLC) – In this topic we discuss this technique that is one of the most used to detect BS.

6.2 Fourier transform infrared spectroscopy (FTIR) – Here is shown the FTIR technique that allows the identification of surfactant compounds and their quantification.

6.3 High pressure liquid chromatography (HPLC) – In this topic is discussed how the BS can be detected in individual peaks and these fractions can be collected for the analysis of the structure of each fraction.

6.4 Tandem mass spectrometry (MS/MS) – The MS/MS technique employs two stages of mass analysis to selectively examine the fragmentation of specific ions in an ion mixture.

6.5 Nuclear magnetic resonance (NMR) – Here we discuss how the NMR helps on the location of each functional group and information on structural isomers in the BS samples.

6.6 Raman spectroscopy – This technique has been used to characterize the structural conformation, functionalities and molecular composition of lipid bilayers and surfactant mono and bilayers.

7 References – Here is listed all the references used for this review paper.

ABSTRACT

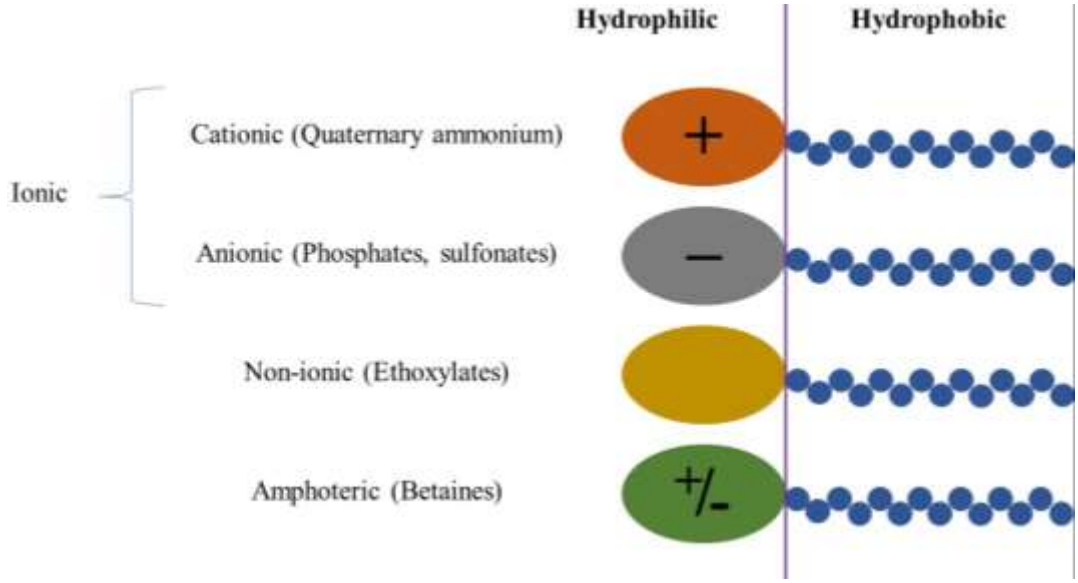
The demand for BS produced by yeast for use in industrial processes and products is increasing. So, there has been an increase in the number of publications related to the characterization of surfactant compounds produced by yeasts with GRAS status, for allowing their applications in several industries, including the pharmaceutical and food industries. However, some of these studies use techniques that are not accurate or are no longer essential, due to the advancement of new technologies. Given the industrial importance of yeasts and their potential to produce BS, this study reviews the production of BS produced by yeasts, their most recent industrial applications, and critically review a wide range of techniques used in the screening of BS producing strains, as well as in the recovery, purification and characterization of these surfactant compounds produced by yeasts. This review introduces the most diverse methodologies that are indispensable for the study of BS produced by yeast, helping in the creation of BS design, synthesis and application, which opens new perspectives in the research of these compounds to overcome the obstacles present in this field.

Keywords: Bioemulsifiers, secondary metabolites, environmental, agricultural, health, medicine.

1 INTRODUCTION

Surfactants are chemical compounds found between aqueous phases with different degrees of polarity and hydrogen bonds (Ribeiro, et al, 2020). These surfactants, depending on their origin, can be synthetic (surfactants) or natural (biosurfactants), and biosurfactants (BS) are composed of amphiphilic molecules with hydrophilic and hydrophobic fractions (López-Prieto et al., 2019). The polar portion can be ionic (cationic or anionic), non-ionic or amphoteric (possess both positive and negative charges depending on the environment in which they are present) and the non-polar portion commonly comprises a hydrocarbon chain (Figure 1). These characteristics give BS the ability to reduce surface and interfacial stress, as well as to form emulsions, which are hydrocarbons solubilized in water or vice versa (Santos et al, 2017).

Figure 1 – Types of surfactants based on polarity of head group.



Source: Modified from Sharma and Oberoi, 2017.

BS have aroused considerable interest in recent decades due to their properties that make them useful in different industrial activities that involve emulsification, detergency, lubrication, foaming, dispersion or solubilization of different phases. In addition, BS have advantages over synthetic surfactants, because they are complex molecules with specific functional groups and have high biodegradability (easily degraded by microorganisms in water and soil), stability and efficacy in a variety of environmental conditions (temperature, pH and salinity), low toxicity and biocompatibility (Rienzo, et al, 2015; Radzuan, et al, 2017; Garg and Priyanka, 2018; Felix et al., 2018).

BS can be used in several industrial sectors, partially or totally replacing chemical surfactants in widely consumed products (Louhasakul, et al 2020), such as laundry detergents (Fei, et al, 2020), household cleaning products and personal hygiene (Perfumo, et al, 2017; Rebello, et al, 2020) and cosmetics (Fernández-Peña, et al, 2020). In the medical field, BS can serve as antimicrobials, antitumor agents and anti-inflammatory agents due to their bioactivity (Fernandes, et al, 2020; Saimmai, et al, 2020). In agriculture, BS can improve soil quality by removing heavy metals and inhibiting the activity of several pests, such as fungi, weeds and insect larvae and nematode that cause drastic reductions in profits (Mnif and Ghribi, 2016). BS also play a significant role in other fields, acting as a larvicidal agent against larvae of the *Aedes aegypt*

mosquito, which causes neglected diseases such as Dengue, Zika and Chicungunha (Marcelino, et al, 2017; Fernandes, et al, 2020), also with the use of BS to stabilize silver and gold nanoparticles, even in the absence of conventional chemical agents (Rane, et al, 2017; Radha, et al, 2019).

Different microorganisms can produce BS, including yeast. Yeasts offer the great advantage of not presenting a risk of toxicity or pathogenicity and, therefore, have the status *Generally Recognized as Safe* (GRAS) (Campos, et al, 2015). These microorganisms can produce BS from oleaginous substrates, including agro-industrial residues, such as glycerol, corn steeping liquor and residual frying oil, which makes the industrial application of BS produced by yeasts viable, reducing production costs (Campos, et al, 2014; Chen et al. 2018).

Considering the reports of the increased use of BS in the most diverse industrial sectors and the growing interest in BS production and characterization, this review discusses the methods of production, detection, classifications, recovery, purification and characterization of BS produced by yeasts, for which there are greater industrial interest. This article also presents an overview for the use of these natural compounds due to their desirable properties.

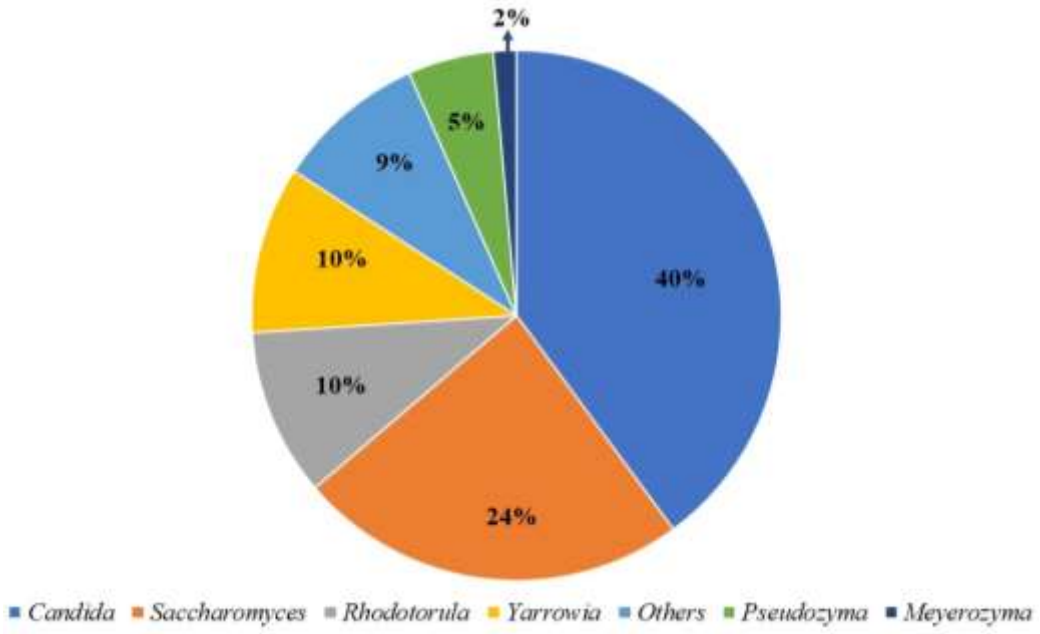
2 BIOSURFACTANTS PRODUCED BY YEAST

Yeasts have gained greater visibility in BS studies on bacteria, as bacterial BS may have restricted use in the pharmaceutical and food industry due to the pathogenic and/or opportunistic characteristics of most bacteria. In addition, yeasts have a higher rate of substrate conversion, producing higher amounts of BS than bacteria (Sharma and Saharan, 2016; Solomon, et al, 2017). Most yeast species also offer the advantage of having GRAS status, as they do not present a risk of toxicity or pathogenicity, allowing their products to be applied in a wide range of industrial sectors (Souza, et al, 2017; Nwaguma, et al, 2019).

In the growing industrial demand for yeasts for several applications (Figure 2), those of the genus *Candida* stand out for the diversity of species producing biosurfactants reported in the literature (Ribeiro, et al, 2020; Camarante, et al, 2021). However, several other yeast genera have been reported recently for the production of BS, such as *Rhodotorula* (Derguine-Mecheri, et al, 2021), *Saccharomyces* (Ribeiro, et al, 2020) and *Wickerhamomyces* (Souza, et al, 2018; Fernandes, et al, 2020) that have potential as producers of BS, exhibiting properties, flocculants,

environmental, antimicrobials, antioxidants and larvicide. In addition, the recovery percentages are higher compared to the yields of BS originating from other microorganisms.

Figure 2 – Publications of the main genera of BS-producing yeasts in the last 5 years.



Caption – Data collected from searches of the number of articles published from 2017 to 2021.

Yeasts are able to grow both on water-immiscible substrates, such as vegetable oils and hydrocarbons, and on water-soluble compounds, such as carbohydrates and glycerol (Souza, et al, 2017; García-Reyes, et al, 2018). The conditions of cultivation and composition of the medium determine the production and composition of the BS, since hydrophobic substrates have a high moisture and protein content that causes their rapid degradation and influences the survival of the producing microorganisms, which use the set of carbon and energy sources for growth. This combination of carbon sources with insoluble substrates facilitates intracellular diffusion because it increases the solubility of water-insoluble compounds and facilitates their transport to the cell (Shekhar, 2015).

One of the first most relevant studies in the production of yeast BS was shown by Pareilleux (1979), in which the yeast *Candida lipolytica* has been shown to produce a complex extracellular polymer with a protein, a lipid and a carbohydrate portion and exhibited emulsifying properties when grown in n-tetradecane or a mixture of linear hydrocarbons. Some years after, Cooper and

Paddock (1984) used two types of carbon sources, carbohydrate and vegetable oil, to obtain large yields of BS from *Candida bombicola* (formerly called *Torulopsis bombicola*) which produced a mixture of glycolipids, which were found by thin layer chromatography showing six components for α-naphthol-positive.

Further research continued to explore the ability of yeasts to produce BS, Ilori et al (2008), evaluated the potential for hydrocarbon degradation and the emulsifying activities of BS produced by two yeast strains, *Saccharomyces cerevisiae* and *Candida albicans*, obtained from a polluted pond. Both strains were able to grow effectively using crude oil and diesel as carbon sources. BS exhibited antimicrobial activities by inhibiting the growth of *Escherichia coli* and *Staphylococcus aureus*. With this work, they demonstrated that these strains represented a new class of BS producers with potential for use in a variety of biotechnological and industrial processes, particularly in the pharmaceutical industry. In the following year, Hirata, et al (2009) showed the production of a glycolipidic BS produced by the yeast *C. bombicula* using soybean oil as a carbon source and their results indicated that this BS produced has low foaming with high detergency, which also presented low cytotoxicity and readily biodegradable properties.

More recently, a research exploring the production of a BS produced by *Rhodotorula* sp., using wastewater from olive mills as a substrate for its production, has been investigated. The isolated yeast strain was considered a potent producer of BS. This BS has been isolated and partially characterized as a complex of glycolipoprotein groups exhibiting interesting properties such as: low critical micellar concentration, significant reduction in surface tension and strong emulsifying activity, the BS produced also proved to have great potential for application in the remobilization of polluted soil hydrocarbons with a removal rate greater than 95% (Derguine-Mecheri, et al, 2021).

This year, another study evaluated the toxicological profile of polymeric nanoparticles encapsulated in polylactic acid-polyethylene glycol (PLA-PEG) biosurfactant in mice. The biosurfactant was isolated from *C. parapsilosis* and was partially characterized by FTIR and GC MS, revealing the presence of a phenol or alcohol group, with the possible presence of an amide in the structure. PLA-PEG copolymer was synthesized using 72 k Da PLA and 6 k Da PEG. The histopathological result of the selected vital organs revealed that BS and/or encapsulated polymeric nanoparticles can be considered safe, since no toxicological characteristics were observed in the

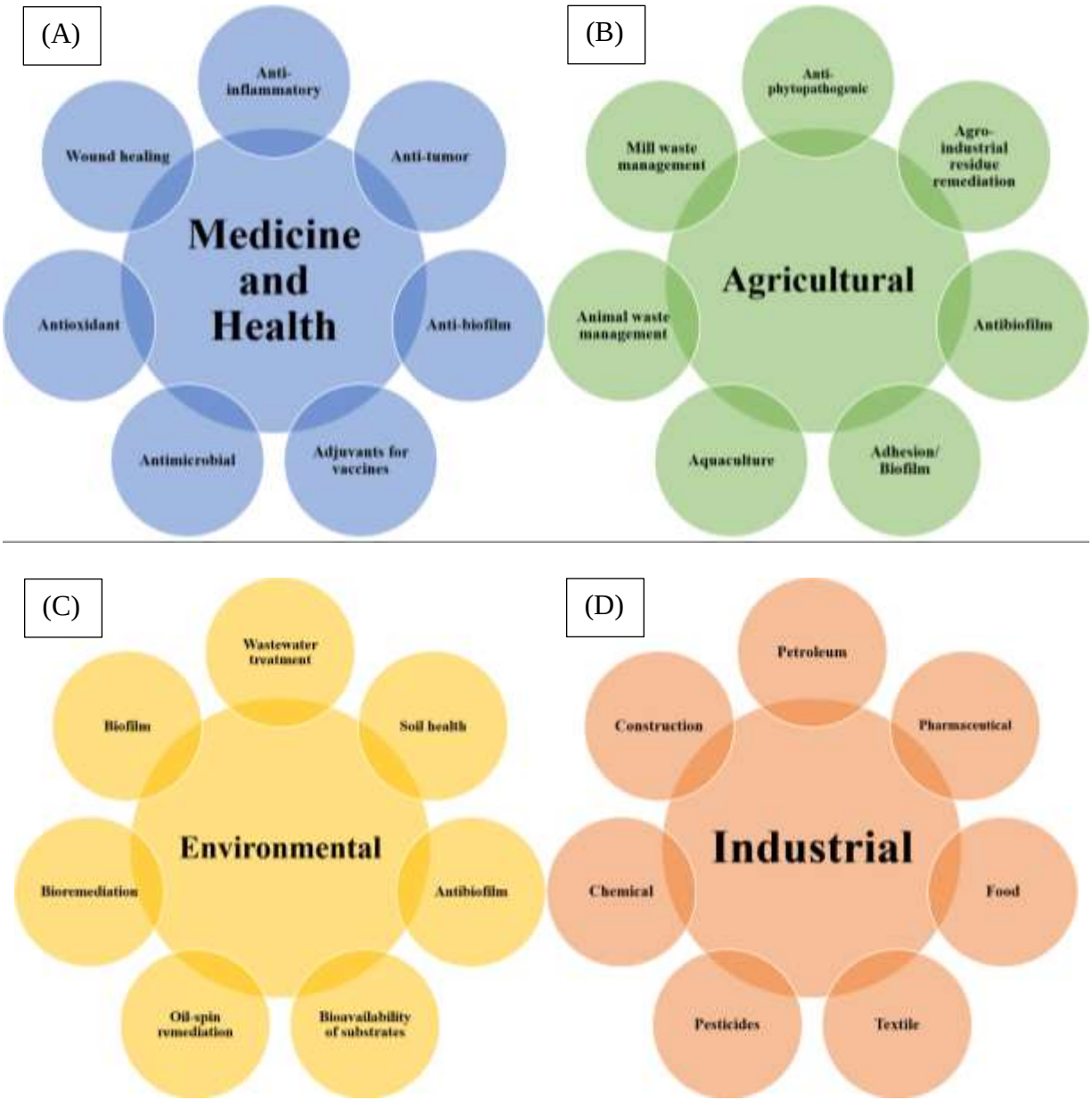
tissue histopathology. Therefore, it can be decided that nanoparticle-encapsulated biosurfactants are non-toxic and can provide a safe and suitable platform for biomedical applications in the future. (Rana, et al, 2021).

In addition to yeast showing high conversions of substrates and being able to have their BS products applied in the food and pharmaceutical industries, as most do not present risks and have GRAS status, it is clear that BS produced by yeasts has been successful in its most diverse applications, from antimicrobials, bioremediations and even in the medical field. With that, the studies of BS produced by yeasts tends to grow more and more, being able to have a competitive market between BS and chemical surfactants.

3 APPLICATION OF BIOSURFACTANTS PRODUCED BY YEAST

The diversity of the chemical structures of BS leads to a variety of functions (Figure 3), which include their interaction with hydrophobic chemicals, due to this capacity, BS influence the absorption, transport and biodegradation of hydrocarbons and xenobiotics, which allows bioremediation applications (Ren, et al, 2018); its ability to form stable micelles, allowing applications in nanoemulsion formulations and other drug delivery systems used against major diseases such as thrombosis, Alzheimer's disease, cancer, etc. (Calabrese, et al, 2017). The amphipathic nature of BS allows them to interact with polar and non-polar surfaces, as well as charged surfaces forming the first layer and acting as wetting agents, this amphipathic ability also assists in microbial adhesion and anti-adhesion at the interfaces, allowing applications on pharmaceutical, agricultural, food and medicine industries (Markande, et al, 2021).

Figure 3 – Properties and applications of Biosurfactants in (A) Medicine/Health sector; (B) Agricultural sector; (C) Environmental sector and (D) Industrial sector.



Source: Modified from Markande, et al, 2021.

3.1 Medicine and Health

BS have emerged as promising molecules due to their structural versatility and diverse properties that can be widely used in the pharmaceutical, medical and cosmetic industries, mainly due to their surface activity and, therefore, can be used as antibacterials, antifungals, antiviral operators, particles safe modulators, antibodies, quality treatment, cancer therapies, as constituents of drug delivery systems or as emulsifiers in cosmetics.

The yeast strains *Candida albicans* and *Candida glabrata* were explored to produce BS which were evaluated for their antibacterial property, the BS of both strains showed antibacterial activity against pathogenic gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*) and gram-negative (*Pseudomonas aeruginosa* and *Escherichia coli*) bacteria at a concentration of 60 mg/L (Gaur, et al, 2019). Previously, the antimicrobial and anti-adhesive activity of BS isolated from *Candida lipolytica* was determined by measuring the percentages of growth inhibition obtained for various microorganisms, it was shown that this BS inhibited the growth of different strains of *Streptococcus* and *Staphylococcus aureus* by more than 90% at one concentration of 12 mg/L. Recently, the BS produced by the yeast *Wickerhamomyces anomalus* exhibited antibacterial properties against *Bacillus cereus*, inhibiting and killing the bacteria at a concentration of 60 mg/L (Fernandes, et al, 2021).

Mannosilitritol lipid (MEL), a glycolipidic BS produced by the yeast of the *Candida* species, has been reported to have neurological and immunological properties along with antimicrobial potential, while the succinyl trehalose lipid produced by *Rhodococcus* species could inhibit certain viruses (Rodrigues, et al, 2006). The yeast *Candida bombicola* could produce a soforolidic BS that exhibited spermicidal activities and was considered hostile to the HIV virus and cytotoxic. The diacetate ethyl ester subsidiary of this soforolipid was the strongest spermicide and virucide in the arrangement of soforolipids examined (Roy, 2017).

Other works can be found in the literature for these applications, such as the one published by Akiyode et al. (2016), which concluded that BS was effective in retarding the growth of the tested cancer cell lines and, therefore, may be potential candidates for use in human cancer therapy. The physicochemical characteristics of BS suggest that its mechanism of action may be due to activity in the cell membrane.

BS produced by yeast also showed larvicidal activity against larvae of the *Aedes aegypt* mosquito, which causes neglected diseases, such as Dengue, Zika and Chikungunya. Marcelino, et al. (2017), showed that the BS produced by the yeast *Scheffersomyces stipitis*, killed 100% of the larvae 12h after the application of BS in the concentrations of 800 and 1000 mg/L. Another study also showed the larvicidal activity of BS produced by *W. anomalus*, in which 100% of deaths were obtained at a low concentration of 63 mg/L in 24h (Fernandes, et al, 2020).

BS can also be used in cosmetics, in the same way as chemically synthesized surfactants and can be used in detergency, emulsification, demulsification, humidification, foaming, dispersion, solubilization of hydrophobic substances or to modify surfaces (Vecino, et al, 2017). Takahashi et al. (2012), investigated the antioxidant properties of different MELs derivatives (A, B and C). All MEL derivatives tested showed antioxidant activity in vitro, but MEL-C, which was produced from soybean oil by *Pseudozyma hubeiensis*, showed the highest rates of elimination of the DPPH radical (50.3% to 10 mg/mL) and elimination of the superoxide anion (50% at 1 mg/mL), had a greater protective activity against oxidative stress and showed the highest antioxidant activity (50.3% at 10 g/L). Based on their results, the authors suggested that MELs have potential as anti-aging ingredients for skin care.

3.2 Agriculture

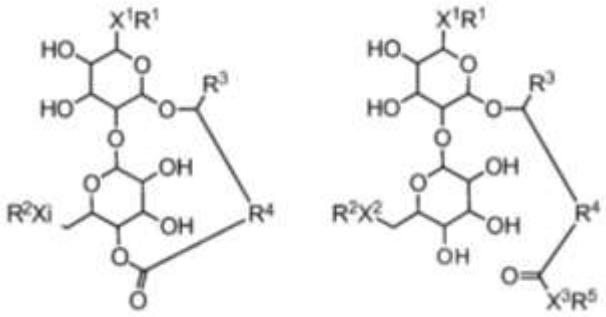
BS can be used as a complement to agribusiness, as they play an important role in helping microorganism products for biocontrol, such as parasitism, antimicrobial, rivalry and protection. In addition, BS can help organisms to adsorb soil particles surrounded by toxins, thus reducing the dispersion path between the assimilation site and the biouptake site by microorganisms. In addition, BS do not have any unfavorable impact on humans or plants and, therefore, can be used in the biological control of plant diseases (Roy, et al, 2017).

Soforolipidic BS, produced by the yeast *Rhodotorula babjevae*, which was isolated from an agricultural field, was evaluated for antifungal activity against phytopathogenic fungi and exhibited promising activity against *Colletotrichum gloeosporioides*, *Fusarium verticilliodes*, *Fusarium oxysporum* and *Trichophyton rubrum*. The *in vitro* antifungal activities of the purified BS were estimated based on the minimum inhibitory concentration (MIC) values and were obtained in the BS concentration range of 62-1000 µg/ml (Sem, et al, 2017).

Another recent work also showed the antifungal activity of BS produced by yeast against species of phytopathogenic fungi, *Aspergillus flavus, Aspergillus niger, Cercospora sorghi, Colletotrichum truncatum, Fusarium verticillioides* and *Fusarium solani*. This BS was produced by the yeast *W. anomalus* and inhibited mycelial growth in up to 95% of all phytopathogenic fungi evaluated in concentrations of 30-50 µg/ml, showing that BS can be applied in agriculture to control diseases and aids in biological control (Fernandes, et al 2020).

A study investigated the application of modified BS (Figure 4), produced by yeast, in plant diseases and showed more evidence for the plant disease control properties of microbial BS. The investigators reported that BS derivatives exhibited significant antifungal activity against 18 phytopathogens (*Alternaria tomatophilia, Alternaria solani, Alternaria alternata, Aspergillus niger, Aureobasidium pullulans, Botrytis cinerea, Chaetomium globosum, Fusarium asiaticum, Fusarium globaminum, Fusarium cereals, Fusarium austroamericana, Fusarium oxysporum, Penicillium chrysogenum, Penicillium digitatum, Penicillium funiculosum, Phytophthora infestans, Phytopthora capsici* and *Ustilago maydis*) and seven bacterial plant pathogens (*Acidovorax carotovorum, Erwinia amylovora, Pseudomonas syringae, Pectobacterium carotovorum, Ralstonia solanacearum, Pseudomonas cichorii* and *Xanthomonas campestris*). The minimum inhibitory concentrations ranged from 0.009 to 10 mg / mL. The same BS were also effective against zoospores of the pathogen *Plasmopara viticola*, which showed loss of viability and lysis occurring at a concentration of 50–500 μg / mL of BS (Schofield, et al, 2013).

Figure 4 – Modified sophorolipids for the inhibition of plant pathogens.



Source: Schofield, Thavasi and Gross, 2013.

3.3 Bioremediation and Oil Recovery

Bioremediation is a process that uses microorganisms for accelerate degradation of environmental contaminants. Biosurfactants increase the surface area, solubility and bioavailability of hydrophobic water-insoluble substrates, stimulating the growth of oil-degrading microorganisms and improve their ability to utilize hydrocarbons (Ron and Rosenberg, 2002).

The crude BS produced by yeast, was evaluated in the removal of heavy metals and petroleum derivatives. The crude BS removed about 96% of Zn and Cu and reduced the concentrations of Pb, Cd and Fe from the specimen. The BS removed 20% of the residual oil using

the permeability apparatus. The results show that BS can be applied in technologies where the removal of heavy metals and petroleum derivatives is desirable. These results demonstrate the versatility of biomolecules of amphipathic nature (Rufino, et al, 2011).

In another study, the BS of *Candida sphaerica* was tested for demulsification of engine oil emulsions, with values around 40%. The crude BS was able to disperse approximately 90% of the oil droplets in seawater and proved to be non-toxic to the native marine microbiota. The present results indicated the potential for applying the BS produced by *C. sphaerica* in the oil industry as a complement to the remediation processes involving contaminated water (Luna, et al, 2015). The same group showed the following year that the BS of *Candida bombicola* also demonstrated the ability to remove oil, obtaining results of 70% of the engine oil adsorbed to the porous surface. The results obtained with the produced biosurfactant showed the promising properties of this biomolecule for use in the bioremediation of hydrophobic compounds (Luna, et al, 2016).

Another study, also involving yeast of the genus *Candida*, analyzed the BS of *Candida lipolytica*. The crude BS did not present toxicity for the bivalve *Anomalocardia brasiliana*, for the microcrustacean *Artemia salina* or for three species of vegetable seeds and stimulated the degradation of motor oil by microorganisms native to seawater. These results indicated that the BS produced has great potential to be applied as a bioremediation agent for cleaning up oil spills, showing the potential to be used not only in the oil industry, but also in other industries (Santos, et al, 2017).

Recently, other studies have also shown the potential for BS produced by yeasts in removing engine oil. Derguine-Mecheri, et al (2021), evaluated the BS produced by *Rhodotorula* sp.YBR and obtained a recovery rate of 98 ± 0.28%. Demonstrating efficient results in the improved removal of hydrophobic contaminants from polluted soils, which makes this BS a promising and potential candidate for environmental applications. In addition, Santos, et al (2021), showed a new formulation of low cost, biodegradable and non-toxic BS by *Candida sphaerica*, in which the dispersion capacity of the engine oil was also investigated. This biomolecule did not show a cytotoxic effect when placed in contact with the L929 cell line, proving to be harmless to the environment. It has also proved effective in removing oil adsorbed on the soil with a dispersion capacity of 90%, presenting potential in bioremediation applications aimed at recovering environments polluted by oily residues.

In addition to applications in bioremediation and removal of heavy metals from soil, another application evaluated by BS produced by yeast was shown in bioleaching assays. *Meyerozyma guilliermondii* showed, in the tests, removal of metal in anaerobic sewage sludge, which was able to solubilize 15.9% of the sewage sludge cadmium (Camargo, et al, 2018).

4 SCREENING METHODS FOR DETECTION OF BIOSURFACTANT

Although several yeast strains are already known to produce BS, many species have been described as new producers of BS and, for that, there are main methods used to screen, detect or evaluate potential BS-producing microorganisms and each has its own advantages and disadvantages (Table 1).

4.1 Hemolytic activity: It is a qualitative screening test for the detection of BS producers. Solid media are used, normally nutrient agar, supplemented with 5% fresh whole blood, called Blood Agar (Banat, 1993). The isolates are seeded and incubated at the required temperature according to the needs of each microorganism for 48 h. Visual inspection for hemolysis may be an indication of lysis of red blood cells due to rupture of the cell membrane caused by the presence of active surface molecules (Figure 4). However, it is very difficult to test the BS productivity of a culture under different conditions directly on the agar (Youssef et al., 2004). Hemolytic activity, however, has been considered an unreliable criterion for the detection of BS activity (Satpute et al., 2008).

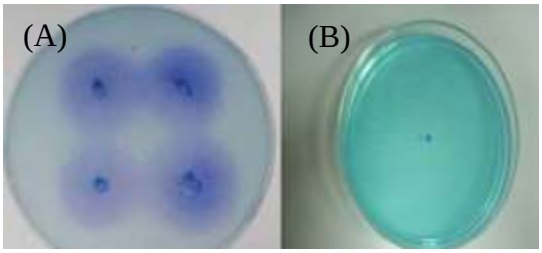
Figure 4 – Hemolytic activity by BS.

Caption – Halo of positive hemolysis. **Source:** Shannaq and Isa, 2013.

4.2 Blue agar plate method: This technique was developed for the detection of glycolipids. The culture medium for this technique, according to Siegmund and Wagner, 1991, uses mineral salt agar (MSA) supplemented with a carbon source (2%), 0.5 mg/ml of cetyltrimethylammonium bromide (CTAB) and 0.2 mg/ml methylene blue (MB). Anionic BS forms a pair of insoluble ions

with the CTAB-MB cationic and the formation of a dark blue halo around the culture is considered positive for the production of BS (Figure 5). It is an excellent technique that has been used generally for the detection of BS glycolipids.

Figure 5 – Blue agar plate method.



Caption – A) BS positive (Dark blue halo); B) Negative control. **Source:** Jamal, et al, 2012.

4.3 Agar plate overlaid with hydrocarbons: The pure isolates are plated on Luria-Bertani agar plates coated with hydrocarbons, such as kerosene, hexadecane, benzene, toluene, diesel or crude oil and incubated for one week at the desired temperature. Colonies surrounded by an emulsified halo are detected as producers of BS (Figure 6) (Morikawa, Ito and Imanaka, 1992). This is an efficient method where the observation of an emulsified halo around the culture is the direct indication of the BS producer.

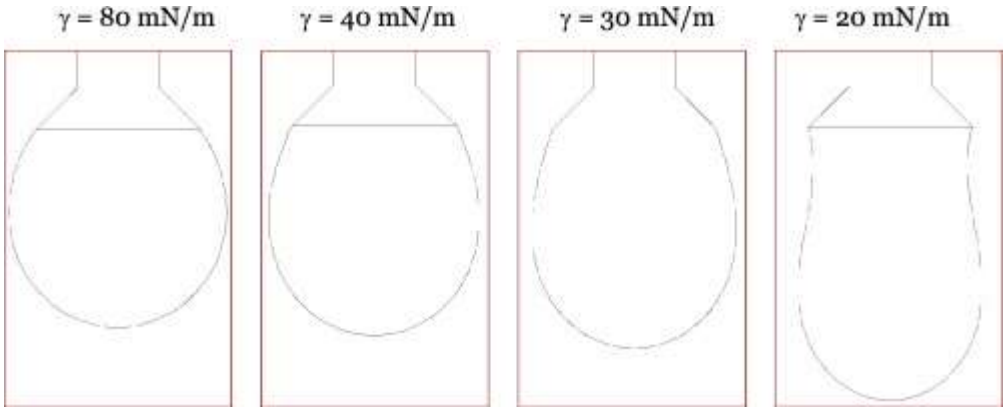
Figure 6 – Agar plate overlaid with hydrocarbons.

Caption – Positive emulsified halo. **Source:** Nayarisseri, et al, 2018.

4.4 Axisymetric drop shape analysis (ADSA): This technique simultaneously determines the contact angle and the surface tension of the liquid from the profile of a drop on a solid surface (Figure 7). The cells are suspended in buffer solution or may be in broth cultures. Consequently, the drop of each suspension is placed on the surface of fluoroethylenepropylene and the profile of

a drop is determined with a counter monitor depending on the time of up to 2 h. The surface stresses of the suspensions are calculated from the droplet profile with ADSA. Only suspensions producing BS show a reduction in surface tension, which depends on the concentration of the product and/or the number of microorganisms producing BS. It is a technique that requires a very small number of cells (Van der Vegt et al., 1991).

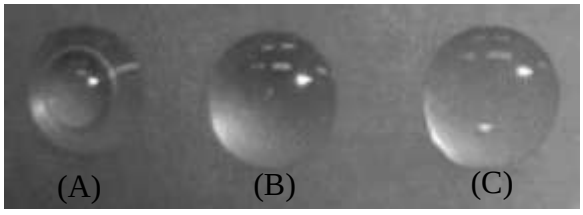
Figure 7 – Axisymetric drop shape analysis.



Caption – Pendant drop shapes with different surface tensions using a 10 µl drop. **Source:** Saad and Neumann, 2016.

4.5 Modified drop collapse method: The microplates are thinly coated with oil. A 5 µL sample of the culture broth is added to the center of the well and observations are made for 1 min. If the drop of a sample collapses with the coated oil, it is an indication of the presence of BS in the culture broth (Figure 8) (Jain et al., 1991; Bodour and Miller-Maier, 1998), being a technique of rapid detection. However, if the sample contains a small amount of BS, false negative results can occur (Satpute et al., 2008).

Figure 8 – Drop collapse method.



Caption – Negative control (Oil + Water); B) Positive control (Oil + Tween 80); C) BS + Oil. **Source:** Nordin, et al, 2013.

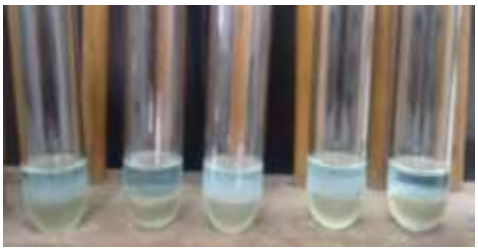
4.6 Oil spreading method: 20 μL of oil is added to 50 mL of distilled water in a Petri dish. 10 μL of the culture broth is added in the middle of the surface of the water coated with oil. If there is an emulsified halo around the culture broth, it is considered positive for the production of BS (Figure 9) (Morikawa, Hirata and Imanaka, 2000). It is one of the fastest methods to detect the presence of BS producers and is considered efficient.

Figure 9 – Oil spreading method.

Caption – Positive emulsified halo. **Source:** Ashitha, et al, 2020.

4.7 Emulsification assay (EA): The culture broth is centrifuged at 10,000 rpm for 15 min and then 3 ml of the supernatant is mixed with 0.5 ml of oil or hydrocarbon and vortexed vigorously for 2 min. The mix is left undisturbed for 1h to separate the aqueous and oily phases (Figure 10) (Jagtap et al., 2009). The absorbance of the aqueous phase is measured using a spectrophotometer at 400 nm and the uninoculated broth is used as a blank. An absorbance of 0.01 units multiplied by the dilution factor is considered to be a unit of emulsification activity per milliliter (Patil and Chopade, 2003).

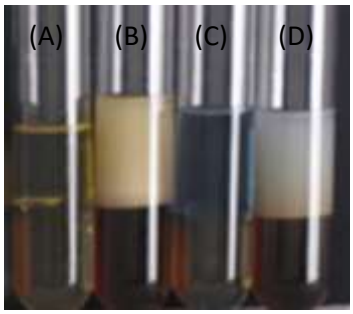
Figure 10 – Emulsification assay.



Caption – Separated aqueous and oily phases. **Source:** Varma, et al, 2017.

4.8 Emulsification index (EI): Emulsification activity is measured by calculating the height of the emulsion for determining EI. This technique is performed by adding kerosene or oil to the culture broth (1:2 v/v). The tube with the sample and kerosene is vortexed for 2 min and left to stand for 24 h. The height of the emulsion is measured in the layers formed between the aqueous layer and the kerosene layer (Figure 11). EI stability designates the strength of a surfactant (Cooper and Goldebberg, 1987).

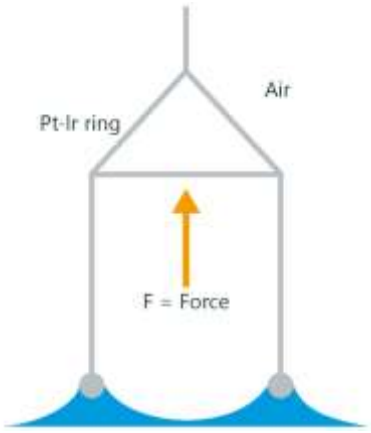
Figure 11 – Emulsification index.



Caption – A) Kerosene – Negative control; B) Emulsification of kerosene by culture supernatant; C) Diesel oil – Negative control; D) Emulsification of diesel oil by culture supernatant. **Source:** Sharma and Saharan, 2014.

4.9 Tensiometric measurement: The measurement of surface tension using a tensiometer is one of the most common methods, in which cell-free supernatant is used. The Wilhelmy plate method, the Du Nouy ring method (Figure 12), the maximum tensile strength method and the slope drop methods are all known for measuring surface tension (Satpute, et al, 2010). The measurement of surface tension is not feasible to apply to a large number of isolates at the preliminary selection level, because the exact measurement of surface tension is difficult to obtain, requiring precise equipment and adequate environmental conditions.

Figure 12 – Du Noüy ring method.



Caption – The force referred to the wetted length acting on a ring as a result of the tension of the withdrawn liquid lamella when moving the ring from one phase to another is measured in this method. **Source:** https://www.kruss-scientific.com/en/know-how/glossary/du-nouey-ring-method.

Table 1 – Screening methods to evaluate potential BS-producing microorganisms and its advantages and disadvantages.

Methods	Advantage	Disadvantage
Hemolytic activity	Easy visualization	The method is not specific; Some biosurfactants do not show any hemolytic activity; Poor specificity of this method; It can give a lot of false negative and false positive results
Blue agar plate method	Easy visualization; Efficient method	It is specific for anionic biosurfactants; The medium can be harmful and inhibits the growth of some microbes.
Agar plate overlaid with hydrocarbons	Easy visualization; Efficient method	It takes a long time (one week) for results

Axisymetric drop shape analysis	Requires a very small number of cells	Equipment is required; Complex calculation; Different samples cannot be measured in parallel.
Modified drop collapse method	Fast and easy method; Requires no specialized equipment and just a small volume of sample	If the sample contains a small amount of BS, false negative results can occur.
Oil spreading method	Fast, easy and efficient method; Requires no specialized equipment and just a small volume of sample	-
Emulsification assay	Simple screening method	Surface activity and emulsification capacity do not always correlate. Consequently, this method gives just an indication on the presence of biosurfactants.
Emulsification index	Simple and efficient method	-
Tensiometric measurement	Accuracy and ease of use	It requires specialized equipment; Measurements of different samples cannot be performed simultaneously.

Chapter – (-) No disadvantage found.

5 RECOVERY AND PURIFICATION OF BIOSURFACTANTS

Although the choice of substrates used in the production of BS has an influence on the general process, productivity and cost of BS, the extraction and purification of BS for a marketable product constitutes 60–80% of the total production costs (Banat, et al, 2014). Therefore, the latest

advances in economic and efficient techniques to recover and purify biosurfactants from the fermentation broth are reviewed in this study (Table 2).

The most common approaches for recovery and purification of BS involve acid precipitation, (Smyth et al, 2014) and extraction of organic solvent, such as ethyl acetate (Banat, et al. 2014), chloroform and methanol (Saikia, et al. 2012). For further purification, different chromatographic methods have been used (Qiao and Shao, 2010; Weber and Zeiner, 2014). However, other methods used to recover BS are also mentioned in this review.

5.1 Acetone precipitation: After fermentation, the cell-free supernatant is mixed with ice-cold acetone to precipitate the emulsifiers, which are then suspended in phosphate buffer. Then, the mixture is incubated at 4°C for 15–20 h to obtain the precipitated BS (Pruthi and Cameotra, 1995). This method was used by several researchers, for the purification of BS (Abouseoud, et al, 2008; Ruangprachaya and Chuenchomrat, 2018; Bhosale, et al, 2019; Vishal, et al, 2020). It is a fast method, relatively inexpensive and without the need for specialized equipment.

5.2 Ethanol precipitation: Like acetone precipitation, ethanol precipitation uses cell-free fermentation broth, which, normally, is mixed with cold ethanol in a 3:1 ratio (ethanol: culture broth). Invally and collaborators (2018), said ethanol precipitation was confirmed not to cause BS loss. It was shown that ethanol precipitation of BS produced by *Acinetobacter calcoaceticus* was the most efficient method when compared to other precipitation methods (Phetrong, et al, 2008).

5.3 Ammonium sulfate precipitation: This method uses $(NH_4)_2SO_4$ for precipitation and is widely used in high molecular weight BS and compounds rich in proteins. This method was introduced by Rosenberg et al. (1979) for precipitation of BS from *Arthrobacter*. For this method, 30% or more of $(NH_4)_2SO_4$ is used added directly to the fermentation broth without removing the cells and left to stand overnight. Subsequently, this precipitate is suspended in 3% saturated $(NH_4)_2SO_4$ and after centrifugation it is added to the $(NH_4)_2SO_4$ supernatant again to reach the final concentration of 40% or desired. The resulting precipitate is centrifuged and extracted with ether. Currently, research uses cell-free broth at 4°C followed by the addition of saturated $(NH_4)_2SO_4$. After cooling, the pellet is resuspended in $(NH_4)_2SO_4$. The pellet obtained after centrifugation is dissolved in water and extracted with an equal volume of hexane to remove residues and the product is subsequently purified by dialysis and lyophilized (Joshi, et al, 2015; Najmi, et al, 2018; Vigneshwaran, et al, 2021).

5.4 Acid precipitation: This method is widely used in the recovery of BS because it is an easy, inexpensive and readily available method. BS is purified from the cell-free supernatant. Acid hydrolysis is carried out using concentrated HCl to lower the pH to 2.0, being left at 4°C overnight. BS becomes insoluble at low pH (Mukherjee, et al, 2006) and proteins and lipids precipitate (Cooper et al., 1981). After cooling overnight, the precipitate is centrifuged and the pellet is subsequently extracted using solvents (Thaniyavarn et al., 2003; Nitschke and Pastore, 2006). The extracted material is filtered to remove residues and evaporated using a rotary evaporator.

5.5 Centrifugation: After acid precipitation, the broth containing BS can also be centrifuged at 12,000 rpm for 15 min at 4 °C to be easily collected as a crude product. Once the pellet is obtained, it can be dried under N_2 and extracted with solvents (Nitschke and Pastore, 2006).

5.6 Crystallization: Once the BS is precipitated/extracted, it is redissolved in an organic solvent. The reaction is also associated with a reduction in temperature, which crystallizes the BS. Therefore, it becomes less soluble in solvents (Manso Pajarron et al., 1993).

5.7 Adsorption-desorption: The interaction of BS molecules with polystyrene resins (XAD 2 or 16) is used for the purification of BS, since their molecules can be adsorbed and desorbed in these resins. The process is initiated by applying cell-free culture broth directly to the adsorbent column, followed by washing with three volumes of demineralized water, to remove unabsorbed compounds. Subsequently, the BS adsorbed and eluted with three volumes of methanol, being removed by evaporation to obtain the crude BS (Gudiña, et al. 2015). In this recently developed approach, BS are adsorbed on polymeric resins and later desorbed with organic solvents. The main advantages of this technique are rapid recovery in one step and obtaining purified BS of high quality.

Dubey et al. (2005) suggested that adsorption-desorption on wood-based activated carbon can be used because the same carbon could be reused for three consecutive cycles for BS adsorption. This process offers good examples of continuous BS recovery from the fermentation broth, using an *in situ* method that reduces the use of high-cost solvents and results in less degradation and prevents product inhibition.

5.8 Foam fractionation: This technique was first reported in 1920 and was used to enrich dissolved compounds (Ostwald, 1920). The basic principle of this technique is the separation of

adsorptive bubbles, so that the air bubbles generated with an aeration system, move to the top of the liquid surface leading to the formation of the foam fraction (Stevenson and Li, 2014). A certain amount of liquid is trapped between the air bubbles and is therefore lost within the foam. However, the force of gravity causes the trapped liquid to drain, which leads to the collapse of the foam. The collapsed foam contains surface and non-surface compounds that are more concentrated than the initial liquid (Beuker, et al, 2014). This technique was previously described and is recently used, being considered a low cost and ecologically correct method, suitable for a process of concentration of products diluted in culture media or extracts (Merz, et al, 2011; Najmi, et al, 2018b).

5.9 Solvent extraction: For this technique, the BS is concentrated from the supernatant by the addition of $ZnCl_2$ and is extracted twice with solvents. The organic phases are evaporated to dryness and analyzed by the TLC technique (Tuleva, Ivanov and Christova, 2002). Hydrophobic portions of BS are soluble in some solvents that aid in the extraction and separation of the crude product. Different solvents, such as chloroform, methanol, ethyl acetate, dichloromethane, butanol, pentane, hexane, acetic acid, diethyl ether, isopropanol are commonly used for the extraction of BS, but these solvents are toxic and expensive. Therefore, it is necessary to use cheap and less toxic solvents for the recovery of BS (Satpute, et al, 2010).

Table 2 – The latest techniques to recover biosurfactants produced by yeasts from the fermentation broth.

Microbial source	Recovery method of BS	Reference
Saccharomyces cerevisiae	Acid precipitation followed by solvent extraction with 1:2 methanol (extract: methanol).	Kreling, et al, 2020
Wickerhamomyces anomalus	Adsorption-desorption chromatography (Amberlite XAD2) using methanol as eluent.	Fernandes, et al, 2020

Saccharomyces cerevisiae	Solvent extraction using ethyl acetate followed by centrifugation and filtration and addition of saturated NaCl and anhydrous $MgSO_4$.	Ribeiro, et al, 2020
Rhodotorula sp.YBR	Acid precipitation followed by solvent extraction with thrice an equal volume of ethyl acetate and methanol (2/1, v/v).	Derguine-Mecheri, et al, 2021
Candida stellata	Ethanol precipitation (500 ml for 1L of broth – 1:2 v/v) followed by centrifugation.	Camarante, et al, 2021
Candida parapsilosis	Acid precipitation followed by centrifugation.	Rana, et al, 2021
Candida sphaerica	Solvent extraction using ethyl acetate (1:1 v/v) followed by other solvent extraction using twice hexane. After extraction, the product was treated with a base and crystallized.	Santos, et al, 2021

6 CHARACTERIZATION OF BIOSURFACTANTS

The use of analytical chemistry techniques to prove the production and characterize surfactant compounds is efficient and conclusive. These techniques include thin layer chromatography (TLC), Fourier transformation infrared spectroscopy (FTIR), high performance liquid chromatography and mass spectrometry (HPLC-MS), mass spectrometry (MS/MS) and nuclear magnetic resonance (NMR). All these techniques require the (partial) purification of surface-active compounds from samples of cell-free supernatants (Twigg, et al, 2020). Different

techniques require different levels of purity of the samples. Therefore, it is necessary to be careful with the purity of each sample to obtain a significant characterization of the surfactant compounds.

6.1 Thin layer chromatography (TLC): TLC is one of the most used techniques to detect BS. The sample is dissolved in solvents appropriate for each type of analysis and applied to the TLC plate which is then placed in a closed chamber with the mobile phase (solvent or mixture of solvents). The mobile phase migrates on the plate and thus, the components also migrate, but at different rates, resulting in the separation. After the race, the spots can be seen in UV light or by chemical treatment (Wall, et al, 2007). To detect BS, the solvent system depends on the type of compound of interest. Organic and inorganic solvents that can be dissolved and are not volatile are preferred. Sometimes, acetic acid, diethyl ether, ethyl acetate, n-hexane, pyridine are necessary for the mobility of BS functional groups (Satpute, et al, 2010). A summary of solvents and treatments for visualizing the spots used for the detection of different BS produced by yeasts is given in Table 3.

Tabela 3 – Solvents and chemical treatments for the visualization of TLC spots used for the characterization of BS produced by yeast.

Microbial source	Mobile phase	Visualization	Reference
Rhodotorula babjevae	Chloroform: methanol: water (65: 15: 2, v/v)	Iodine fumes and Anthrone reagent	Sen, et al, 2017
Scheffersomyces stipitis	Chloroform: methanol: distilled water (65:15:1 v/v)	Seebach reagent	Marcelino, et al, 2017
Cyberlindnera saturnus	n-hexane: ethyl acetate (5:3 v/v) and Acetonitrile: methanol: water (4:2:1 v/v)	UV light, Ninhydrin reagent, Anthrone reagent and Rhodamine 6G reagent	Balan, et al, 2019
Cutaneotrichosporon mucoides	Chroform: methanol (19:1 v/ v)	Seebach reagent	Marcelino, et al, 2019
Rhodotorula sp.YBR	Acetone: acetic acid: water (70:20:10 v/v) for amino acids; Chloroform:	Iodine fumes, Ninhydrin reagent and Molisch reagent	Derguine-Mecheri, et al, 2021

	methanol: water (60:30:10) for sugars and Chloroform: methanol: water (65:25:10 v/v) for lipids		
Wickerhamomyces anomalus	Acetonitrile: metanol (3:1 v/v)	UV light and p-Anisaldehyde	Fernandes, et al, 2021

6.2 Fourier transform infrared spectroscopy (FTIR): FTIR has been proposed as a rapid technique that allows the identification of surfactant compounds and their quantification (Leitermann, et al, 2008). However, it should not use only FTIR to conclusively identify the type of surfactant compound produced by a microorganism. As an FTIR principle, the chemical bonds present in the compound produce a specific spectrum that can be detected when analyzing a sample by comparison with a known pattern of a specific compound or with patterns that have analogous chemical groups (Leitermann, et al, 2008). However, as these chemical bonds are not exclusive to surfactant compounds and can be present in many other extracellular compounds produced by microorganisms, the sample must therefore have a high level of purity to allow this detection to be specific (Twigg, et al, 2020). This technique has been used in a series of studies that report the production of BS by yeast strains (Radha, et al, 2019; Ribeiro, et al, 2020; Camarante, et al, 2021).

6.3 High pressure liquid chromatography (HPLC): This technique consists of a stationary phase (a solid column, over which the mobile phase continuously flows the components of the sample solution), mobile phase (carries the injected sample solution through the injector port) and a detector (emits a response due to sample elution and subsequently signals a peak in the chromatogram). In this technique, the components migrate according to the non-covalent interactions of the compound with the column, this separation being based on its polarity and its separate products can be detected in individual peaks and these fractions can be collected for the analysis of the structure of each fraction. (Haba, et al, 2003; Satpute, et al, 2010). The BS can be separated and identified successively when the HPLC is coupled to an evaporative light scattering detector (ELSD) or when mass spectrometry (MS) is employed. HPLC along with the installation of MS are important to provide the molecular mass of each fraction (Smyth, et al, 2014).

6.4 Tandem mass spectrometry (MS/MS): The MS/MS technique employs two stages of mass analysis to selectively examine the fragmentation of specific ions in an ion mixture. The types of instruments that can be used to perform this experiment are based on the separation of mass analysis events over time or based on measurements on physically separate analyzers (De Hoffmann, 1996). In the MS/MS technique, a single congener can be fragmented, and the resultant daughter ions analyzed (Tripathi et al., 2019). This technique provides more precision for the characterization of BS compounds being produced by microbial strains (Twigg, et al, 2020) and has recently been used to provide confirmatory evidence of BS production by the species of yeast *W. anomalus*, by direct injection of a solution in an electrospray ionization source in a quadrupole ion-trap mass spectrometer, in positive and negative mode. The tandem mass spectra for the most intense ions to carry the mass proportions were in the range of 400-1000 and the MS spectra in positive mode shown major peaks at 682 and 828 m/z. According to the MS direct infusion, no important peaks were detected in in negative mode, which means that no important free acidic group was present in the biosurfactant molecule (Fernandes, et al, 2021).

6.5 Nuclear magnetic resonance (NMR): This technique is based on transitions of atoms with a magnetic moment when an external magnetic field is applied, and the absorbance of radiation by a nucleus in a strong magnetic field. The absorption of radiation causes the nuclear spin to realign or rotate in the direction of higher energy, as soon as the energy is absorbed, the nuclei will reissue radiation and return to the low energy state. The transition energy of NMR depends entirely on the intensity of the magnetic field and a factor of proportionality for each nucleus called the magnetogyric ratio. NMR provides information about the functional groups, as well as the position of the bonds within the carbohydrate and lipid molecules. The exact location of each functional group can be obtained and information on structural isomers is also possible with the help of a series of NMR experiments. Solvents such as acetic acid, acetone, benzene, chloroform, dimethyl sulfoxide, methanol, pyridine and water are used. BS must be dissolved and applied to a series of 1D (1H and 13C) and 2D (such as COZY, TOCSY, HMQC and HMBC) experiments by NMR and analyzed, this technique has been used to determine chemical structures of BS since 1960 (Tulloch et al, 1968; Asmer et al, 1988; Agrawal, 1992; Satpute, et al, 2010).

Detailed analyzes of BS produced by yeast have been performed with the help of NMR and have recently been reported in the literature. Fernandes et al, 2021, isolated a BS produced by

W. anomalus and used the technique to characterize the crude BS and its fractions, which were dissolved in chloroform / methanol (3: 1 v / v) and the analyzes were performed using a 600 NMR spectrometer. The spectrum of the crude biosurfactant in ^{13}C NMR showed the presence of double bonds with signals at 130 ppm. In addition, several CH_2 groups resonated at 22.79 - 34.35 ppm and two signals at 14.15 and 14.19 corresponding to two primary methyl groups (CH_3). The spectrum also revealed C-O bond signals at 63.4, 65.18 and 65.43 ppm, which could be from the glycerol chain. No signal was resonated close to 110 ppm (C1 sugar carbon). The ^{13}C NMR spectrum of the crude biosurfactant revealed at least three peaks at 177 ppm, typical of carbonyl groups.

6.6 Raman spectroscopy: In biological and chemical systems, Raman spectroscopy has been used successfully to characterize the structural conformation, functionalities and molecular composition of lipid bilayers and surfactant mono and bilayers, including the interdigitization of lipid and surfactant chains or tail groups in bilayers (Suga, et al, 1995; Suna, et al, 2013).

Recently, purified BS extracted from *Pseudozyma* yeast strains were characterized by traditional Raman scattering and surface-enhanced Raman scattering methods and exhibited similar vibrations in the regions of 2700−2800 cm^{-1} and 2850−3050 cm^{1}, which correspond respectively to asymmetric and symmetric C-H stretching vibrations of terminal CH_2 and CH_3 groups. This work demonstrated that Raman spectroscopy can be a marker-free and efficient method to identify BS and differentiate the level of saturation in the acid chain (Leu, et al, 2020).

7 FINAL CONSIDERATIONS

Effective screening methodologies and improved purification techniques play a vital role in obtaining a better quality biosurfactant and greater quantity. Biosurfactants produced by yeasts have been shown to be effective in applications in several industrial areas. Using biosurfactants produced by yeasts, the possibilities for these applications in the pharmaceutical and food industries increase, in addition to yeasts being better producers than bacteria, since they produce a greater amount of BS in less time. Currently, the commercial success of biosurfactants is limited by the high cost of production, so optimized conditions for growth and production, the use of renewable and economically viable substrates and the use of microorganisms with higher substrate conversion rates would help to produce more profitable and economically viable biosurfactants.

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CHAPTER IV

Analysis of metagenomic data for microbiome studies and identification of bacteria present in tea tissue culture (*Camellia sinensis*)

1 INTRODUTION

Tea is the second most consumed non-alcoholic beverage in the world, behind only water, with important economic, health and cultural values (Xia et al., 2017). It is produced from the leaves of the tea plant - an evergreen crop planted globally that belongs to the *Camellia* genus of the *Theaceae* family (Mondal et al., 2004). Tea has abundant characteristic metabolites, such as polyphenols, caffeine, amino acids, vitamins and minerals beneficial to human health (da Silva, 2013). Currently, the tea plant has been introduced in more than 50 countries worldwide for large-scale commercial cultivation. More than three billion people drink tea in more than 160 countries (Xia, et al, 2019). According to United Nations Food and Agriculture Organization (FAO, 2020) statistics, the world's tea plantation area has exceeded 4.1 million hectares and more than 5.95 million metric tons of tea worldwide are produced annually.

Bacterial contamination is a serious problem in plant cultures *in vitro*, both in the micropropagation of commercial plants, as it hinders the start of the culture, reducing the efficiency of multiplication and rooting of the shoots, and in research laboratories, where contamination can be the causative agent of false results in physiological experiments (Orl Ikowska, et al. 2010). The diversity and abundance of exobiotic and endobiotic bacteria genera and species that accompany donor plants (Leifert, et al. 1991) is a major challenge in the sterilization of initial explants, rapid detection of bacteria in the first passages *in vitro* and minimization of its adverse effects on seedling multiplication and rooting efficiency. In most cases, bacteria are introduced into the cultures together with the initial explants (Kałużna, et al. 2013).

In practice, the initial explants are only sterilized on the surface and, thus, live microorganisms internally are introduced into *in vitro* cultures. If symptoms of bacteria colonizing plant tissues appear in a short time, contaminated explants should be removed immediately. If bacterial growth is very slow or temporarily retarded under plant growing conditions, they remain in a cryptic state and may appear only when the growing conditions will change drastically, for

example, after delayed subculture, temperature rise, change in the composition of the medium or due to other factors (Thomas, 2004).

However, the plant-microorganism association can present benefits that may vary depending on the specific characters of the bacteria and interactions with the plant. Some bacteria can produce not only all the hormones produced by plants (Friesen et al. 2013), but also other growth regulators, unusual for plants, that can affect plant morphogenesis and development. Vereecke et al. (2000) reported that the supernatant of the *Rhodococcus fascians* culture medium, which causes distortions both *in vivo* and *in vitro* within the plants (galls, stem fasciation, brooms) contained 11 distinct cytokines, which interfered in the metabolism of the plants, influencing even the plants morphogenesis of *in vitro* cultures. Therefore, it is important to know the microorganisms that may or may not be present in tissue culture.

Based on the characteristics of in vitro cultivation of *Camellia sinensis* and the benefits of this crop for human health, this study aimed to identify the bacteria present in tea tissue culture through metagenomics in California, United States and such a way, establish a possible improvement in *in vitro* cultivation of that crop.

2 MATERIAIS E METODOS

2.1 Plant material and in vitro culture conditions

For the present study, the buds of tea (*Camellia sinensis*) were collected in greenhouse from mother plants K13 and K14 and placed in plastic bags and transported to the laboratory. Sterilization was carried out according to the following protocol: 3 minutes in 70% ethanol in slow agitation, followed by 20 min in 20% sodium hypochlorite in slow agitation and then washed 3 times in sterile distilled water.

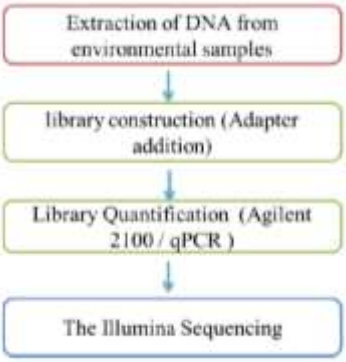
After sterilization, the buds were cut and all anthers were removed and placed in plates with sterile B5 culture medium (B5 3.21 g/L, Sucrose 30 g/L, 2.0 mg/L NAA, 0.2 mg/L BAP, 400 mg/L Carbenicillin, 250mg/L Cefotaxime, 150 mg/L Timentin, 2mLs/L PPM, 8 g/L Phytoagar) and incubated at 28^{o}C in the dark for approximately 6 weeks, or until the appearance of callus. The calluses were removed and placed in plant tissue culture cups with sterile B5 medium for the formation of new plants. The plants were grown in the Plant Biology Laboratory until approximately 15 cm of growth and then the whole plant was collected for metagenomic analysis.

2.2 Shotgun metagenomic

2.2.1 Library construction and sequencing

For Shotgun metagenomic sequencing, quality control (QC) was performed at each stage of the procedure to maintain data reliability. The DNA of the samples was extracted. Agarose gel electrophoresis was used for DNA purity and integrity, and quantification by Qubit 2.0 flowmeter was used for accurate measurement of DNA concentration. Physical fractionation was applicable by Covaris Sonicator. The fractionation steps were verified by Agilent2100 and Q-PCR to ensure that sufficient enrichment of the target was achieved. The sequencing was performed using the Illumina platform after grouping the library with readings of paired ends. The workflow is as follows:

Figure 1 – The workflow of metagenomic library construction.



2.2.2 Bioinformatic Analysis

Assembly of the metagenome: The metagenome was assembled based on clean data after the quality control of each sample and put the unused readings of each sample together for mixed assembly to explore the information of species of low abundance of the samples.

Prediction of the gene: The prediction of the gene was performed by MetaGeneMark based on the scaftigs that were assembled by simple and mixed samples. Group the genes planned for re-replication to build the gene catalog. Based on the clean data for each sample in the gene catalog, abundance information from the gene catalog for each sample was obtained.

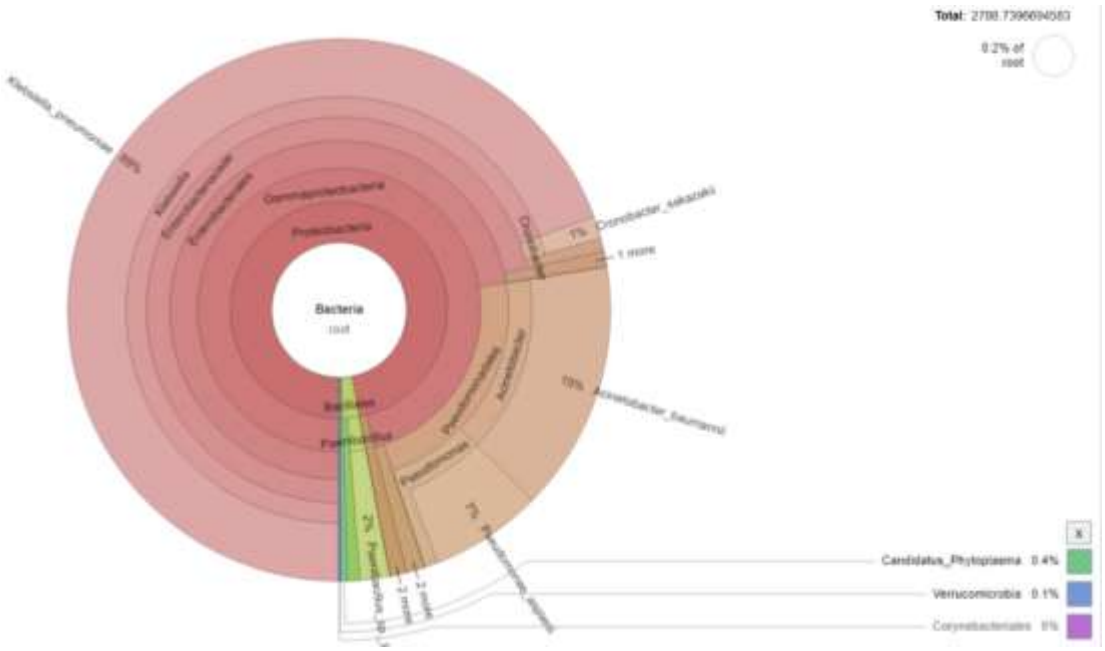
Taxonomy annotation: This procedure involved comparing metagenomic readings with the database of taxonomically informative gene families (NR database) to annotate each

metagenomic counterpart. The abundance tables of different taxonomic classifications were based on the gene abundance table.

3 RESULTS AND DISCUSSION

Two varieties of *Camelia sinensis* (K13 and K14) were chosen for the analysis of metagenomics to identify bacteria present in these cultures *in vitro*. For both varieties, 0.2% of bacterial DNA from the total extracted DNA was found. Within this percentage, K13 presented 97% for the phylum *Proteobacteria*, including *Klebisiella pneumoniae* (69%), *Acinetobacter baumannii* (15%), *Pseudomonas asplenii* (7%) and 3% for the order *Bacillales*, which includes *Paenibacillus* sp.IHB_B_3415 (2 %) and *Staphylococcus aureus* (0.7%). The genus Candidatus Phytoplasma was also shown in the K13 sample, exhibiting 0.4% of the total bacterial DNA. The phylum with the least amount of bacterial DNA in K13 was *Verrumicrobia*, exhibiting only 0.1%, as shown in Figure 2.

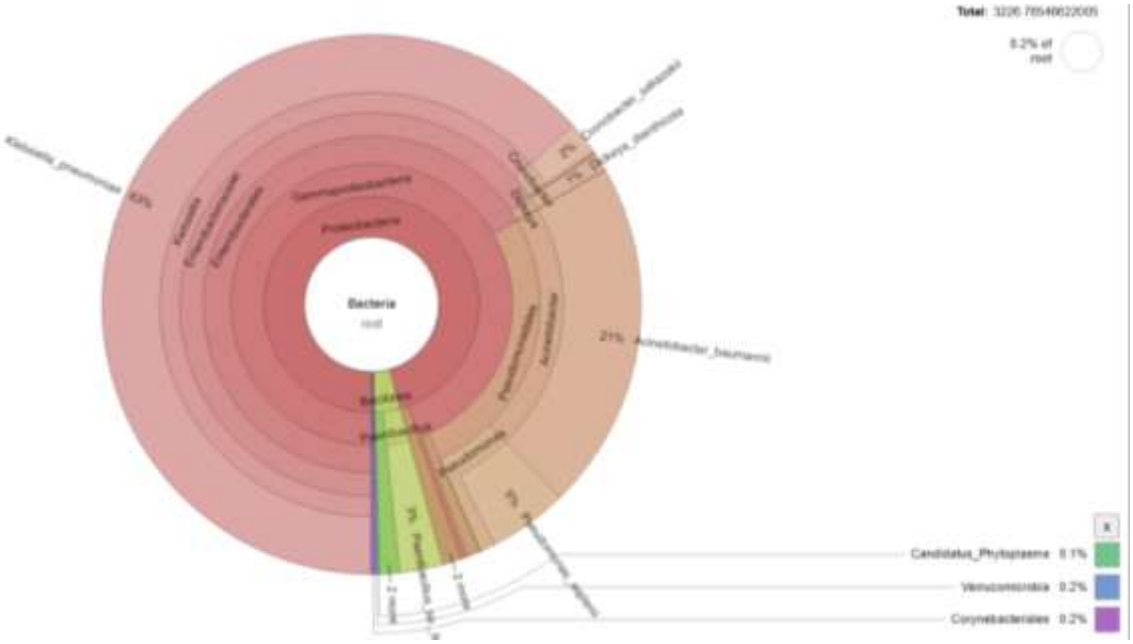
Figure 2 – Interactive Krona for K13 Metagenomics.



For K14, the phylum Proteobacteria showed a concentration of 96% of the total bacterial DNA found in the sample, including the bacteria *Klebisiella pneumoniae* (63%), *Acinetobacter baumannii* (21%), *Pseudomonas asplenii* (5%) and presented 4% of DNA bacterial for the order *Bacillales*, with the bacteria *Paenibacillus* sp.IHB_B_3415 (3%), *Staphylococcus aureus* (0.8%)

and *Listeria monocytogenes* (0.3%). With the lowest concentrations of bacterial DNA, 0.2% were found for the phylum *Verrumicrobia*, 0.2% for the genus *Corynebacteriales* and 0.1% for the group Candidatus Phytoplasma (Figure 3).

Figure 3 – Interactive Krona for K14 Metagenomics.



Bora, et al. (2021), evaluated the microbial population in *C. sinensis* plants and showed that the distribution at the class level, that the rhizosphere was highly populated by *Proteobacteria* (40.98%), *Acidobacteria* (19.74%), *Firmicutes* (14.43%), *Actinobacteria* (10.95%), *Bacteroidetes* (4.70%), *Verrucomicrobia* (3.05%) and *Cyanobacteria* (1.9%); while the endosphere was inhabited by *Proteobacteria* (89.17%), *Actinobacteria* (5.08%), *Bacteroidetes* (1.52%), *Firmicutes* and *Synergistetes* (both with 0.85%). These results were similar to what was found here, although it was not done in tissue culture, they showed that the majority of the bacterial population of *C. sinensis* plants are *Proteobacteria*. Most of these bacteria have been reported to either promote plant growth or protect the plants from pests and diseases (Fernández-González et al. 2017; Verma et al. 2019).

Another study, using the independent cultivation technique, metagenomics, showed that *C. sinensis* plants were colonized predominantly by *Proteobacteria* (46.9–56.1%) and *Bacteroidetes* (40.1– 53.0%) (Cernava, et al, 2019). Confirming that the phylum *Proteobacteria* is common in tea plants. Sun, et al (2019), evaluated the presence of bacteria present in tea tissue culture, to find

bacteria that contribute to the production of theanine by the plant. The endophyte isolated from *in vitro* cultures of *C. sinensis* cultivars was also identified as a *Proteobacteria*, which showed strong biocatalytic activity for conversion of glutamine and ethylamine to theanine. Corroborating with the present work and showing that the bacteria of the phylum *Proteobacteria* can also be found in in vitro tea cultures.

Table 1 shows the species and genera found in each of the varieties of tea tissue culture. In total, 10 species and 3 genera were found in the K13 variety and 12 species and 3 genera in the K14 variety, with 8 species and 2 genera common among the varieties.

Table 1 – Bacterial DNA from metagenomics results of varieties K13 and K14.

K13	K14
Klebsiella pneumoniae	*Klebsiella pneumoniae*
Cronobacter sakazakii	*Cronobacter sakazakii*
Dickeya dianthicola	*Dickeya dianthicola*
Acinetobacter baumannii	*Acinetobacter baumannii*
Pseudomonas asplenii	*Pseudomonas asplenii*
Pasteurella multocida	*Pasteurella multocida*
Staphylococcus aureus	*Staphylococcus aureus*
Aster yellows phytoplasma	*Aster yellows phytoplasma*
Verrucomicrobia	*Verrucomicrobia*
Paenibacillus sp.IHB_B_3415	*Paenibacillus* sp. IHB_B_3415
Escherichia coli	*Candidatus* Paraburkholderia *schumannianae*
Escherichia sp._R8	*Acetobacter malorum*
Vibrio cholerae	*Agrobacterium rhizogenes*
	Listeria monocytogenes
	Mycobacteriaceae

Caption – Shaded species and genera were found in both varieties.

Although the disinfection of materials from donor plants is always of special importance in the micropropagation process, contamination is usually introduced with the initial explants. Bacteria live in plant vessels, intercellular spaces, but also within plant cells (Pirttilä, et al. 2000), which may be one of the reasons for the failure of surface sterilization. However, not every plant-bacterial association is bad and some of these microorganisms may be contributing positively to the success of the propagation and growth of the plant.

Klebsiella pneumoniae was the most abundant within 0.2% of total extracted DNA, with 69% dominance. Inigez, et al. (2004), showed that *K. pneumoniae* relieved the symptoms of nitrogen (N) deficiency and increased the total concentration of N in the plant. The nitrogen fixation was confirmed by the dilution of the ^{15}N isotope in the plant tissue and in a plant product, chlorophyll. In this context, nitrogen is particularly important as an essential macroelement for normal plant growth and development, which represents about 1.5% to 2% of the plant's dry matter and approximately 16% of the total plant proteins (Scheible, et al. 2004). The assimilation of N is related to the main physiological or metabolic processes, such as photosynthesis, photorespiration, respiration, amino acid synthesis and tricarboxylic acid cycle (Foyer, et al. 2011). Therefore, despite being a human pathogen, bacteria *K. pneumoniae* can present a positive factor for plant nutrition and growth.

Chronobacter sakazakii, was a bacterium found in both samples and, despite being a human pathogen, Schmid, et al. (2009) showed that the strains of *Cronobacter* spp. showed characteristics frequently found in microorganisms associated with plants and rhizosphere, including solubilization of mineral phosphate, indole acetic acid and siderophore production. In addition, the ability to colonize tomato and corn roots endophytically has also been demonstrated. The results provided evidence that the plants may be the natural habitat of *Cronobacter* spp and may also be a positive relationship between microorganism-plant. The other bacteria found may not show benefits, some are known to be plant pathogens, and can be harmful to the development and growth of plants.

4 CONCLUSIONS

It was showed here that most of the bacterial population of the *C. sinensis in vitro* plants are *Proteobacteria*. Most of these bacteria have been reported to either promote plant growth or protect the plants from pests and diseases. Although the disinfection of materials from donor plants is always of special importance in the micropropagation process, contamination is usually introduced with the initial explants. But not every plant-bacterial association is bad and some of these microorganisms may be contributing positively to the success of the propagation and growth of the plant. However, more studies need to be done to investigate the origin of these contaminants to avoid them or use them as a benefit.

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