# Methods of purification and characterization of biosurfactants: an overview

## Abstract

Biosurfactants are amphiphilic molecules produced by several microorganisms including bacteria, filamentous fungi and yeasts. They are classified according to their chemical composition (glycolipids, lipopeptides, glycoproteins, glycolipopeptides, phospholipids) and their molecular weight (low and high molecular weight). Biosurfactants are currently used in several industrial fields (food industry, pharmacy, medicine, detergents, agriculture, cosmetics, oil recovery and bioremediation) owning to some of their specific properties such as their low toxicity, biocompatibility, biodegradability, environmentally-friendly, stability under extreme conditions (temperature, pH and salinity), structural diversity, production using renewable low-cost substrates, agreement with green chemistry and sustainability. Despite the properties of biosurfactants, there are few studies leading to their structural characterization. The most reported structures are from biosurfactants produced by *Bacillus* spp. (surfactins, fengycins, lichenysines) and *Pseudomonas* spp. (rhamnolipids). The majority of researches performed on biosurfactants emphasized their functional groups characterization. These biosurfactants have yet to be fully characterized at structural level. Hence, in this review, we highlight the different purification and structural characterization techniques which can be combined to provide information on the structure of the biosurfactants independently of its complexity. This will lead to enhance their application in some highly pointed industrial fields such as medicine and pharmacy.

**Keywords**: Biosurfactants, Microorganisms, Purification, Structural characterization.

## Introduction

Biosurfactants are amphiphilic surface-active compounds produced by microorganisms from diverse sources (terrestrial, marine environments, sludge, etc.) including bacteria, molds and yeasts (Nataraj *et al*., 2021a; Mouafo *et al*., 2022). They are either excreted extracellularly or remain bound to the producing cells (Vallejo *et al*., 2021). They are synthetized by microorganisms through different metabolism routes depending of the nature and the composition of the substrates (Figure 1). Once the substrates are intake, some microorganisms produced enzymes that might combine the hydrophilic and hydrophobic components of the substrates leading to the formation of biosurfactants. The substrates can also be metabolized into coenzyme into acetyl coenzyme A, that will be used in *de novo* pathway as the precursor of the synthesis of sugars, lipids and proteins (Tokumoto *et al*., 2009). Hence, the enzymatic combination of these hydrophilic (sugar, proteins) and hydrophobic (lipids) components synthetized by the microorganisms will lead to the biosurfactants. In some case, the hydrophilic (sugar, proteins) or hydrophobic (lipids) components synthetized by the microorganisms through *de novo* pathway can be combined with carbohydrates, proteins or lipids' components intake from substrates (Sydatk and Wagner, 1987). According to Desai and Banat (1997), microorganisms often used water insoluble substrates for the synthesis of the hydrophobic moiety of the biosurfactants and the water soluble ones for the synthesis of the hydrophilic moiety. Biosurfactants are classified according to their chemical composition and their molecular weight. With regards to the chemical composition, they can be glycolipids (rhamnolipids, Mannosylerythritol lipids, trehalolipids, xylolipids), lipopeptides (surfactins, viscosines, fengycins, lichenysines), glycolipopeptides, glycoproteins, phospholipids, neutral lipids, polymeric biosurfactants (emulsan, alasan, biodispersan), and particular biosurfactants (protein–sugar–lipid complex molecules) (Bergevin *et al*., 2017; Nitschke and Silva, 2018; Mouafo *et al*., 2022). Considering the molecular weight, biosurfactants are grouped as low molecular weight (glycolipids, lipopeptides, phospholipids) and high molecular weight (particular biosurfactants, polymeric biosurfactants) compounds (Smyth *et al*., 2014).

Several investigations on the potential replacements of synthetic surfactants by biosurfactants have been conducted this last decade. The main reasons that have impelled these investigations are their low toxicity, biodegradability, environmentally friendly, biocompatibility, structural diversity, stability under extreme conditions of temperature, salinity and pH, production using renewable low-cost substrates, agreement with green chemistry and sustainability (Marchant and Banat, 2012a; Marchant and Banat, 2012b; Mouafo *et al*., 2018a; Nitschke and Silva, 2018). These advantages of biosurfactants associated with their diverse properties (emulsifying, de-emulsifying, foaming, dispersing, wetting, solubilizing, antioxidant, antiadhesive, antimicrobial and antibiofilm agents) confer to these latter's, the ability to be applied in many industrial fields including, cosmetics, detergents, oil recovery and bioremediation, agriculture, pesticides, pharmaceuticals/medicines, and food industry (Nitschke and Silva, 2018; Silva *et al*., 2020; Patel *et al*., 2021; Mouafo and Somashekar, 2020; Mouafo *et al*., 2022). This justifies the increasing demand of biosurfactants for which the markets reached 2210.5 million dollars by 2018 (Transparency Market Research, 2013) and the high number of companies (approximately 17) around the world, which currently produced biosurfactants (Randhawa and Rahman, 2014; Sekon and Rahman, 2014).

The activity and structure of biosurfactants which derived from the secondary metabolism of substrates by microorganisms, strictly depend on the producing strain, the medium composition, and the culture conditions (Mulligan *et al*., 2014; Liu *et al*., 2015). Although in the literature there are some studies on the full structural characterization of biosurfactants, the majority of these researches concerned lipopeptides' biosurfactants derived from *Bacillus* spp. (surfactins, fengycins, utirins, lichenysins) and glycolipid biosurfactants derived from *Pseudomonas* spp. (rhamnolipids) (Joshi *et al*., 2016; Sharma *et al*., 2016; Abdalsadiq *et al*., 2018). For the other microorganisms, almost researches performed on biosurfactants emphasized their functional groups characterization (Lara *et al*., 2020; Nataraj *et al*., 2021a, Nataraj *et al*., 2021b; Sakr *et al*., 2021; Vallejo *et al*., 2021; Mouafo *et al*., 2022). These biosurfactants have yet to be fully characterized at structural level. The principal hurdles are the complex nature of some biosurfactants particularly those derived from lactic acid bacteria (Mouafo *et al*., 2022). Hence, several purification and characterization techniques are needed to obtain structural information on the molecule. In this review, we highlight the different purification techniques reported in the literature as well as the different colorimetric, spectrophotometric, chromatographic and spectrometric methods which can be combined to obtain the structure of the biosurfactants independently of its complexity. Relevant data used in this review were from several original articles, review articles, book chapters and books. They are were obtained from PubMed, EMBASE, Google Scholar, Scopus, Google, Web of Science and Google. The search terms were the combination of these four keywords: “biosurfactants”, “techniques”, “purification” and “structural characterization”.

## 1. Purification techniques of biosurfactants

Several techniques were reported in the literature concerning the purification process of biosurfactants. These techniques included dialysis, membrane filtration, centrifugation, acid precipitation, thin layer chromatography and column chromatography. Currently, no defined commercial method is available for the purification of the biosurfactants. Table 1 summarizes the different methods of purification of biosurfactants according to the producing strain et the nature of the biosurfactant.

### *1.1. Centrifugation and ultracentrifugation*

Biosurfactants extracted for culture media are complex mixture of several compounds. The centrifugation allows the separation of these compounds based on their density even tiny the difference can be. According to the size of biosurfactants, higher gravitational-force instruments or ultra-centrifuges are very often used. That technique has been widely used for purification of biosurfactants. A centrifugation at high speed (13,000×g, 4°C, 20 min) was used by Mouafo *et al.* (2020) to separate the biosurfactants produced by *L. casei* subsp. casei TM1B from contaminants. A centrifugation at 10,000 rpm, 4 °C, 15 min was applied by Madhu and Paprulla (2013) in the purification process of biosurfactants. It is important to mention that centrifugation and ultracentrifugation are not used as lone purification techniques of biosurfactants. They are always used as a unit operation of a process that included several other unit operations

### *1.2. Precipitation*

Almost biosurfactants are known for their low solubility under acidic conditions (Nelson et al., 2020). That property was exploited by several authors to purify biosurfactants from other contaminants. Madhu and Paprulla (2013) acidified the solution of crude biosurfactants to pH 2 for 18 h, and used acidic water (pH 2.0) to wash the precipitate in order to remove possible contaminants. The precipitation of biosurfactants was also achieved using ice-cold acetone (Bhosale *et al*., 2019), chilled ethanol (Fookao *et al*., 2021; Devale *et al*., 2023) and ammonium sulphate (Vigneshwaran *et al*., 2021).

### *1.3. Chromatography*

Generally, biosurfactants are produced by microorganisms in association with other molecules. These molecules can be extracted together with biosurfactants following the extraction method chosen. Hence there is a need for purification and chromatography appears a one of the main methods. By the means of the mobile phase the mixture of compounds is flowing through the stationary phase and they are separated. The separation process is based on the differential partitioning of compounds between the mobile and stationary phases. The most reported chromatographic methods for biosurfactants purification are column chromatography, thin layer chromatography (TLC) and reverse phase chromatography (Rodrigues *et al*., 2006; Sauvageau *et al*., 2012; Mouafo *et al.*, 2020).

### *1.3.1. Thin layer chromatography*

Based on the properties of the biosurfactants present in the mixture, a solvent is chosen. That solvent into which biosurfactants should be dissolved will move up the plate the spot of the compounds deposited at the bottom of the plate. Depending on the physical properties and the structure of compounds from the mixture, they will move up the plate or stays behind. Then, spots are visualized in UV light or after chemical treatment. The solvent systems generally used for purification of biosurfactants on TLC plates are: petroleum ether/diethyl ether/acetic acid, acetone/acetic acid/water, chloroform/methanol/water, ethyl acetate/methanol/water, chloroform/methanol/water/acetic acid (Sauvageau *et al.*, 2012; Banerjee and Ghosh, 2021; Meena *et al*., 2021, Dabaghi *et al.*, 2023; Fernandes *et al*., 2023).

### *1.3.2. Column chromatography*

That technique separates a complex mixture of biosurfactants based on their molecular weight, size and their affinity with the stationary phase. The stationary phases used are generally either silica gel (60–120 mesh) or sephadex, while mobile phase consists of a mixture of solvents with different polarities. Rodrigues *et al*. (2006) used an octyl Sepharose 4 FF Prep hydrophobic interaction column and a linear gradient of PBS buffer containing 1.0 M $(NH_4)_2SO_4$ for elution of the complex mixture of biosurfactants from *Streptococcus thermophilus* A. Thavasi *et al.* (2011) and Sharma and Saharan (2016) used silica a gel (60–120 mesh) column with a gradient solvent system (chloroform/methanol) starting from 20:1 to 2:1 (v/v). Reverse phase HPLC using a C-18 column with a gradient solvent system (trifluoroacetic acid/millipore water, 0.05:99.95 and TFA/millipore water/acetonitrile, 0.05:19.95:80) was applied by for purification of biosurfactants from *L. casei* subsp. casei TM1B (Mouafo *et al*., 2020). A silica gel column (60 Mesh) with chloroform–methanol-water (65:25:4) as mobile phase was successfully used by Banerjee and Ghosh (2021) to purify biosurfactants from *Bacillus oceanisediminis* H2. A Sephadex G-25 column with the sodium phosphate buffer (20 mM; pH 7.5) as elution solvent was used by to purify biosurfactants from *B. velezensis* KLP2016 (Meena *et al.,* 2021). Biosurfactants from *B. subtilis* LSFM-05 was purified on a silica gel column (0.03–0.07 mm, 60 A°) using the following solvent system at different polarities: chloroform, methanol, and an aqueous solution of 28% (v/v) ammonium hydroxide (de Faria *et al*., 2021). Vigneshwaran *et al.* (2021) purified biosurfactant from *Brevibacillus* sp. AVN13 using fast protein liquid chromatography.

However, in almost reported studies, the collected active fractions following column chromatography were pooled by the authors (Thavasi *et al.*, 2011; Sharma and Saharan, 2016). This might reduce the probability to discovery new biosurfactants.

### *1.4. Filtration*

This method uses the difference in pressure between two sides of a special membrane to separate biosurfactants from other molecules according to their size. Depending on the membrane pores' size and the pressure, it can be microfiltration, ultrafiltration or nanofiltration (Satpute *et al*., 2010). The crude biosurfactants derived from *L. acidophilus* NCIM 2903 and *L. casei* subsp. casei TM1B was purified from contaminants using filtration through a 0.22 µm membrane pore size (Satpute *et al*., 2019; Mouafo *et al*., 2020).

### *1.5. Dialysis*

As a good method for sensitive compounds, dialysis enable separation of biosurfactants from accompanying substances simultaneous extracted with biosurfactants such as salts. In the dialysis process, molecules driven by a differential concentration gradient, are transported through a semipermeable membrane based on their size. Hence, contaminants present in the crude biosurfactants will pass through the semipermeable membrane leading to the purification of this latter. In the purification process of biosurfactants, Vecino *et al.* (2017), Ghasemi *et al.* (2018), Satpute *et al.* (2019) and Vigneshwaran *et al.* (2021) used a membrane of molecular weight cutoff 6000–8000 Da to perform dialysis against double demineralized water. The process has led to the obtention of pure biosurfactants.

Globally, the choice of the purification techniques of biosurfactants depends on the producing strain, the culture media and the extraction method. There are some microorganisms that produce only one molecule of biosurfactant as major metabolism compound (*Bacillus* spp., *Pseudomonas* spp.). Depending on the production mode (extracellular or cell-bound), the biosurfactants is extracted from the culture media. The great challenge in the purification process is to separate the biosurfactants from the culture media or cells' components which can be extracted together with the biosurfactants. However, other microorganisms (lactic acid bacteria) produced complex mixture of molecules with different features as biosurfactants. In this case, the choice of the purification technique becomes difficult and requests that several techniques should be used with special care to the screening of desired activity during the process. Hence, at least three different techniques should be used in combination with TLC or column chromatography to strengthen the purity of the biosurfactants (Mouafo *et al.*, 2022).

## 2. Physicochemical characterization of biosurfactants

### *2.1. Identification of ionic properties of biosurfactants*

The different types of biosurfactants vary according to their biochemical composition. Based on their large structural diversity, the biosurfactants might be charged or not. This has led to the classification of biosurfactants depending on their ionic properties. Hence, the different classes are: anionic, cationic, amphoteric and non-ionic. The CTAB agar method was developed and was generally used to detect the production of biosurfactants of glycolipidic nature (Satpute *et al.*, 2008). The method is based on the fact that the biosurfactants can form an insoluble ion pair (precipitation lines) with the cationic cetyl trimethyl ammonium bromide (CTAB) or anionic sodium dodecyl sulfate (SDS). Methylene blue is sometimes added in the culture media to ease the observation of the precipitation line materialized as a blue halo around well containing culture (Satpute *et al.*, 2008). This method was successfully used to confirm the anionic nature of biosurfactants derived from *Lactococcus lactis* (Saravanakumari and Mani 2010) *E. faecium* (Sharma *et al*., 2015), and *Pseudomonas guguanensis* strain Iraqi ZG.K.M (Faisal *et al*., 2023). However, the method is not suitable for other types of biosurfactants such as lipopeptides or glycoproteins.

### *2.2. Color of biosurfactants*

The color of biosurfactants generally varies according to the producing strain, the culture media composition and the extraction technique. The colors reported in the literature were either whitish, yellowish or brownish (Faisal *et al*., 2023). Fig. 2 presents the color of biosurfactants produced by some lactobacilli strains (Mouafo, 2019).

### *2.3. Identification of chemical nature*

#### *2.3.1. Screening of proteins*

The reaction of ninhydrin and amino acids is used to screen the presence of proteins in biosurfactants. After TLC plates development, plates are dried, sprayed with ninhydrin solution and kept at 90°C for 30 min. Appearance of pink or red spots indicates the presence of amino acids and thus reveals the proteinaceous nature of the biosurfactants (Banerjee and Ghosh, 2021; Faisal *et al*., 2023).

#### *2.3.2. Screening of sugars*

At high temperature and in presence of α-naphthol and sulfuric acid, carbohydrates reacted, leading to the formation of a pink complex. That reaction was exploited to screen the presence of carbohydrates in biosurfactants. Plates obtained following TLC development of complex mixture of biosurfactants are sprayed with α-naphthol solution and concentrated sulfuric acid and heated at 100°C for 5 min. Appearance of pink spots indicates the presence of carbohydrates in biosurfactants (Satpute *et al*., 2010). That method was applied directly on a solution of pure biosurfactants by Atta *et al.* (2009). The authors confirmed the presence of carbohydrates in the biosurfactants after the apparition of a violet or purple colour between the two layers formed by the solution of biosurfactants and the reagents. Sen *et al*. (2017) reported that the TLC plates can be sprayed with anthrone reagent for detection of sugars while Satpute *et al.* (2019) noticed that sugars detection can be assessed through TLC plates spraying with diphenylamine. In the studies of Reddy *et al*. (2016), orcinol reagent was sprayed on TLC plates to detect the presence of sugars in biosurfactants.

### *2.3.3. Screening of lipids*

The presence of lipids in biosurfactants is generally assessed on TLC plates. The principle is based on the reaction between iodine vapors and lipids in a close chamber that leads to the development of a yellow color (Sen *et al*., 2017; Faisal *et al*., 2023). The method is not specific as a positive result might indicate the presence of neutral or polar lipids (Atta *et al*., 2009). According to de Faria *et al*. (2021) and Meena *et al*. (2021), the presence of lipids in biosurfactants can be assessed through the spraying of TLC plates with water followed with drying. Appearance of white spots on the TLC plates indicates the lipophilic nature of the biosurfactants.

### *2.3.4. Screening of phosphate groups*

Some biosurfactants were reported as containing phosphate groups in their constitution (Rodrigues *et al*., 2006). A rapid method to assess phospholipids in biosurfactants was developed by Okpokwasili and Ibiene (2006). In that method, a solution of biosurfactants is mixed with nitric acid 6M, the mixture is heated at 70°C for 30 min and a solution of ammonium molybdate 5% (w/v) is added drop by drop. Apparition of a yellow color and formation of a yellow precipitate at the bottom of the tube indicate the presence of phospholipids.

### *2.4. Identification of the chemical composition*

#### *2.4.1. Protein content*

In the literature, the protein content of biosurfactants was quantified through different methods. The dye-binding method of Bradford (1976) was used by Ghasemi *et al.* (2019) and Behzadnia *et al*. (2020) to assess the protein content of biosurfactants. In that method, the Coomassie G-250 dye is bound to proteins leading to the formation of a blue complex that absorbs at 595 nm. The dye-binding method of Bradford is relatively rapid and suitable for determination of protein content of biosurfactants with high molecular weight (Smyth *et al*., 2010). In the study of Morais *et al*. (2017), the method of Lowry *et al*. (1951) was used to quantify protein in biosurfactants. That method combines the Biuret reagent (used to assess protein through the presence of peptide bonds) and the Folin-Ciocalteau phenol reagent (used to assess the residues of tryptophan and tyrosine).  The intensity of the blue colored complex developed is measured spectrophotometrically at 660 nm. The Kjeldahl method (AOAC, 1990) was used by Mouafo *et al.* (2018) to assess the protein content of biosurfactants derived from three lactobacilli strains. In that method, biosurfactant was digested with a strong acid leading to the release of nitrogen which was titrated. The nitrogen content was then converted to protein content using an appropriate conversion factor.

#### *2.4.2. Lipid content*

Lipids are one of the major constituents of biosurfactants. They are mostly responsible for the amphiphilic nature of biosurfactants. The lipid contents of biosurfactants were assessed using the method of Folch *et al*. (1957) by Ferreira *et al*. (2017) and Ghasemi *et al*. (2019) [57]. That method based on the solubility of lipids in solvents of different polarities. When solvent is heated, it evaporated, passed through the sample, extracted lipids and carried these later into the flask where they can be quantified after solvent evaporation. However, as lipids are always complexed in biosurfactants with proteins (lipoproteins), carbohydrates (glycolipids) or the association of proteins and carbohydrates (glycolipoproteins) (Mouafo *et al*., 2022), the most suitable method to assess their content required to break the bond that hold lipids prior solvent extraction. Generally, hydrolysis with HCl 3N at 100°C for 1 h is used to release these bound lipids (Morais *et al*., 2017).

### *2.4.3. Sugar content*

Sugars are important elements for the composition of some types of biosurfactants such as glycolipids, glycoproteins, and glycolipoproteins. They can be simple sugars, oligosaccharides, or polysaccharides. The most reliable method widely used in the literature to assess the sugar content of biosurfactants is the phenol–sulfuric acid of Dubois *et al*. (1956). In that method, hydrolyzed saccharides of biosurfactants are dehydrated into furfural derivatives while reacting with concentrated sulfuric acid. The furfural derivatives obtained reacted with phenol to form a colored complex that absorb light at 490 nm. The phenol–sulfuric acid was successfully used by Ferreira *et al*. (2017), Mouafo *et al*. (2018a), Mouafo *et al*. (2018b), Behzadnia *et al*. (2020), Mouafo *et al*. (2020) and Devale *et al*. (2023) while identifying the chemical nature of biosurfactants.

## 2.5. Elemental composition of biosurfactants

Element analysis provides information that can be used to determine the chemical nature and structure of biosurfactants. The elemental composition of biosurfactants was assessed in the literature with different equipment. Rodrigues *et al*. (2006), used X-ray photoelectron spectroscopy (XPS) to determine the percentage of C, N, O and P in biosurfactants from

*Streptococcus thermophilus* A. Based on the higher amount of C and O, the authors concluded on the glycolipid nature of the biosurfactants. The energy dispersive X-ray was successfully used by Habib *et al.* (2023) to assess the elemental composition of biosurfactants from *P. benzoelyticum* Pb4, *Bacillus albus* S2i and *Proteus mirabilis* Th1. Ferreira *et al*. (2017), and Vecino *et al.* (2017), used a Carlo Erba EA-1108CHNS-O element analyzer to determine the percentage of C, N, H and S in the biosurfactants from *L. paracasei*. The authors reported respectively the glycoprotein and glycolipopeptide natures of biosurfactants based on their C, N, H and S contents. The glycolipoprotein nature of biosurfactants derived from *L. paracasei* subsp. tolerans N2 was identified by Mouafo *et al*. (2018b) using a Vario EL III elemental analyzer associated with biochemical analyses.

## 3. Identification of functional groups of biosurfactants

### *3.1. FTIR spectroscopy*

Fourier transform infrared spectroscopy (FTIR) is a method widely used for the characterization of biosurfactants. It generally leads to determine the chemical nature of biosurfactants based on the functional groups and chemical bonds that are present in the biosurfactants (Jemil *et al*., 2017). In the method, biosurfactants are submitted to radiation ranging from 400 cm$^{-1}$ to 4000 cm$^{-1}$. When the radiation frequency is close to the resonance frequency of the biosurfactants that is analyzed, there is absorption of the luminous energy. A decrease of the transmitted energy is then observed. The absorption bands obtained that vary according to chemical bonds and functional groups of constituent elements of the biosurfactants are characteristic of the studied biosurfactants. FTIR was used by several authors to determine the chemical nature of biosurfactants (Morais *et al*., 2017; Vecino *et al*., 2017; Mouafo *et al*., 2018b; Mouafo *et al*., 2020; Banerjee and Ghosh, 2021; Dabaghi *et al*., 2023; Faisal *et al*., 2023; Habib *et al*., 2023). It is generally performed by 32 scans of the compounds at a spectral resolution of 4 cm$^{-1}$ and a wave number accuracy between 400 and 4,000 cm$^{-1}$. Background reference used is always potassium bromide pellets.

### *3.2. Ultraviolet spectroscopy*

Several microorganisms produced biosurfactants thus leading to a broad structural diversity. They are mixture of lipids (saturated or unsaturated fatty acids), proteins and sugars that possess several kinds of bonds which can absorb ultraviolet (UV) light in the range of 100 to 800 nm (Wilcox and Wilcox, 1995; Sakr et al., 2021). The ultraviolet spectroscopy method was used by Dehghan-Noudeh *et al*. (2005) and Ismail *et al*. (2013) to identify the lipopeptidic nature of biosurfactants produced by *Bacillus* sp. Authors reported peaks at 215 nm corresponding to α and β-unsaturated ketones, peaks at 260 nm corresponding to aromatic rings thus indicating the presence of aromatic amino acids. Based on these information's, they concluded on the lipopeptidic nature of the biosurfactants. Sakr *et al*. (2021) reported that biosurfactants from *L. plantarum* 60FHE, *L. paracasei* 75FHE, and *L. paracasei* 77FHE absorbed in the far UV region (270-277 nm) and were all glycolipopeptides.

### *3.3. Fatty acids profile of biosurfactants*

The structural diversity of biosurfactants leads to a great variation in their lipid moiety. To obtain detailed structural information on that lipid moiety, the different fatty acid entering in their constitution should be identified. The most suitable methods for that are GC-FID (gas chromatography coupled with flame ionization detection) and GC-MS (gas chromatography coupled mass spectrometry). In these methods, the lipid moiety of biosurfactants is prior separated from the other moiety through a hydrolytic process that will cleave the link (Smyth *et al*., 2010). The fatty acids obtained are transformed into a volatile derivate (fatty acid methyl esters) which will be easily analyzed in GC-FID using standards or in GC-MS. Fatty acid methyl esters are separated according to their chain length, and their molecular mass registered provide information on their structures. The fatty acids profile of biosurfactants were determined by several authors in the literature using GC-MS (Sharma *et al.*, 2015; Morais *et al.*, 2017; Vecino *et al.*, 2017; Behzadnia *et al.*, 2020; De Gregorio *et al.*, 2020; Mouafo *et al.*, 2020 ; Faisal *et al.*, 2023).

### *3.4. Monosaccharide's profile of biosurfactants*

The sugar moiety of biosurfactants is always made of monosaccharides bound through glycosidic links. To identify these monosaccharides, the first step is the separation of the sugar moiety of the biosurfactants from the other moiety which could be lipids, proteins, or both depending on the nature of the biosurfactants. That separation is generally carried out through hydrolysis trifluoroacetic in a sealed tube at 120°C for 4-6 h. Then, the mixture is neutralized with 2 M $NH_4OH$, reduced with an aqueous solution of sodium borohydride ($NaBH_4$) and then, the sugars are transformed into volatile derivates (N-trimethylsilylimidazole, alditol acetates). The obtained derivates are injected in GC-MS which will provide structural information. The sugar moieties of biosurfactants were successfully identified by Sauvageau *et al.* (2012), Morais *et al.* (2017) and Mouafo *et al.* (2020) using GC-MS.

### *3.5. Amino acids profile of biosurfactants*

#### *3.5.1. Amino acids profile*

Amino acids are constitutive units of peptides that entering into the composition of several class of biosurfactants such as lipopeptides, glycopeptides and glycolipopeptides. The peptide moiety of biosurfactants is linked to other compounds through amide and lactone bonds (for fatty acids chain) or N-glycosidic and O-glycosidic bonds (for sugars) (Joshi *et al.*, 2016). Prior identification of the amino acids of the peptide moiety of the biosurfactants, these bonds should be cloven. That cleavage generally occurs after hydrolysis at high temperature (110°C) with concentrated HCl (6 N) for approximately 24 h in sealed tubes. The amino acid obtained can be analyzed by GC-MS after conversion into volatile derivatives (trimethylsilylation). That method provides information on the amino acid composition of the biosurfactants based on the retention times and masses (m/z). GC-MS was successfully used by You et al. (2015) to identify four amino acids (Val, Leu, Asp and Glu) in the lipopeptide from *Enterobacter* sp. N18 after acid hydrolysis and trimethylsilylation. However, that method does not provide information on the sequence of amino acids.

### *3.5.2. Amino acid sequence*

Amino acids sequence of biosurfactants can be assessed using Edman degradation or Quadruple-time-of-flight tandem mass spectrum (Q-TOF MS/MS). Edman degradation is the earlier method reported in the literature to elucidate the composition and sequence of amino acids of biosurfactants (Smyth *et al*., 2014). The method was mostly applied for lipopeptides. In that method, the biosurfactant is hydrolyzed and the smaller cleaved peptides are submitted to Edman degradation. Mild alkaline hydrolysis is generally applied to open the ring of lipopeptides. The Edman degradation method required peptides from the biosurfactants to be purified to homogeneity (Smyth *et al*., 2014). The Edman degradation process is conducted in an automated sequencers and the cleaved amino acids are submitted to chromatography. The retention time of the cleaved amino acid is compared with the one of amino acid standards for identification (Zachara and Gooley, 2000).

Opposite to the Edman degradation technique, Q-TOF MS/MS is applied directly on the entire biosurfactants without a preliminary hydrolysis step (You *et al*., 2015). In that method, the initial mass (m/z) of the biosurfactants is determined. Then, amino acids from biosurfactants are lost sequentially leading to a decrease of its m/z after MS/MS fragmentation. The m/z lost corresponds to the mass of the amino acid eliminated. The C-term amino acid is often identified using the double hydrogen transfer mechanism of Yang et al. (2006). In that mechanism, the m/z of the C-term amino acid is increased of m/z value of 18 which correspond to the m/z of water. Direct infusion in the mass spectrometer or application of liquid chromatography (LC, HPLC, UPLC) leading to separation of individual peptides before mass spectrometer analysis can be used. The method is less time consuming and required small volume of samples (Smyth *et al*., 2014). Korenblum *et al*. (2012) used Q-TOF MS/MS to identify Glu-Leu-Leu-Val-Asp-Leu-Leu as the amino acid sequence of biosurfactants produced by *Bacillus* sp. H2O-1. The peptide sequence Gly–Ser–Thr–Leu–Leu–Ser–Leu–Leu was identified from the biosurfactants produced by *P. fluorescens*. BD5 using MALDI TOF/TOF mass spectrometry and MS/MS fragmentation (Janek *et al*., 2010). The same method was used by Pereira et al. (2013) to identify the sequence of the heptapeptide moiety (L-Glu-L-Leu-D-Leu-L-Val-L-Asp-D-Leu-L-Leu) of the biosurfactants from three strains of *B. subtilis*. Fragments indicating the sequential losses of the amino acid residues Leu/Leu/Asp/Val/Leu/Leu/Glu-OMe was identified in the biosurfactants from *B. subtilis* LSFM-05 by de Faria et al. (2011) while using Fourier transform ion cyclotron resonance mass spectrometry with electrospray ionization (ESI-FTICR-MS).

## 4. Molecular weight of biosurfactants

The method commonly used to assess the molecular weight of biosurfactants is Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE). The method is recommended for high molecular weight biosurfactants (Smyth *et al*., 2010). In that method, biosurfactants are separated in a gel load with a reducing buffer and SDS. They will be separated according to their molecular mass (Toren *et al*., 2001). Fouad *et al*. (2010) have successfully determined the molecular weight of surlactin (a glycolipid biosurfactants) produced by *L. acidophilus* using SDS-PAGE. The molecular weight of the protein fraction of biosurfactants derived *L. acidophilus* NCIM 2903 was also determined by Satpute *et al*. (2019) using SDS-PAGE. Tricine-SDS-PAGE was performed by Vigneshwaran *et al*. (2021) to identify the molecular mass the biosurfactant *Brevibacillus* sp. AVN13

## 5. Mass spectrum of biosurfactants

Mass spectrometers provide information on the structure of biosurfactants through an identification of all constitutive elements of the molecule. The method identifies both the moieties of the biosurfactants without a preliminary hydrolysis. The MS spectra provides information on the mass (m/z) of the protonated molecules $[M+H]^+$ of and their sodium $[M+Na]^+$ or potassium $[M+K]^+$adducts. During MS/MS fragmentation, constitutive elements of the biosurfactants are released and identified based on the m/z values. The direct infusion to the mass spectrometer and injection into liquid chromatography (LC) connected to a MS detector system, so as 90% of each fraction is collected and 10% sent to MS detector, are generally used. The only condition is the purity of the biosurfactants. For this purpose, it is always recommended to perform and HLPC-UV or HPLC-DAD to confirm the purify of the compound with the appearance of a well-constructed and differentiated peaks. These peaks correspond to pure fractions. HPLC-ESI-MS, LC-MS/MS, Tandem MS/MS, HRMS and other mass spectrometry techniques were used by several authors to determine the structure of biosurfactants (de Faria *et al*., 2011; Janek *et al*., 2013; Pereira *et al*., 2013; You *et al*., 2015; Oluwaseun *et al*., 2017; Abdalsadiq *et al*., 2018; Adu *et al*., 2023).

## 6. Nuclear magnetic resonance of biosurfactants

NMR is an analytic technique that consists to measure the absorption of radio frequencies by atoms of biosurfactants submitted to a magnetic field. That magnetic field allows the resonance of atoms present in the molecule. The different resonance frequencies of the atoms are consigned on a graph which permits to determine the structure of the molecule based on chemical shifts (Abdel-Mawgoud *et al*., 2011). Briefly, NMR required a principal static magnetic field and a magnetic field that oscillates in the field of radio frequencies ($10^6$-$10^7$ Hz). Sample is dissolved in solvents (deuterated chloroform, methanol pyridine, acetic acid, dimethyl sulfoxide acetone, benzene) and introduced into an induction coil that generates an oscillating magnetic field. Depending on the nucleus present in the sample and the value of the principal static magnetic field, the sample will absorb and reemit energy in a particular way and at a precise frequency named resonance. Some recent NMR spectrometers are equipped with triple resonance helium-cooled TCI cryoprobe ($^{1}$H, $^{13}$C and $^{15}$N or $^{31}$P) and provide information on the chemical environment of atoms of H, C, N, and P within the biosurfactants (Hamerly *et al*., 2015).

Proton and carbon NMR are amongst the most used techniques for structure elucidation of biosurfactants. It was used by several authors independently of the biosurfactants producing strain (Monteiro *et al*., 2007; Sauvageau *et al*., 2012; Pereira *et al*., 2013; Sharma *et al*., 2015 ; Oluwaseun *et al*., 2017; Mouafo *et al*., 2020 ; Dabaghi *et al*., 2023). In the process of identifying the exact position of constitutive atoms (carbons, hydrogen, nitrogen, phosphorus) of the biosurfactants with respect of their chemical environment, two dimensions NMR (COSY, HMBC, ROSY, and HSQC) is often used to strengthen information provided by one dimension $^{1}$H and $^{13}$C NMR (Sauvageau *et al*., 2012).

## 7. Successful structure elucidation of biosurfactants.

Successful characterization leading to the proposition of a chemical structure to biosurfactants was achieved by some authors in the literature. Globally, the combination of at least two techniques depending on the biosurfactants and the producing strains, was required.

Nelson *et al.* (2020) used HPLC coupled to high-resolution electrospray ionization mass spectrometry to characterize the biosurfactants from *L. sakei* JN-185, *L. fermentum* JN-119 and *L. plantarum* JN-141. They identified several compounds including glycosyldiglycerides, surfactin C13, iturin A8, octapeptin D, plantaricin A, lichenysin A, sakacin-A, glysperin, plusbacin A3 and laterocin. The biosurfactants from *Pseudomonas putida* BD2 was identified by Janek *et al*. (2013) using TLC and UPLC/ESI-MS/MS. They found that the biosurfactants was a mixture of di-rhamnolipid (Rha-Rha-C10-C10) and phosphatidylethanolamines PE

(32:1), PE (33:1). You *et al*. (2015) used the combination of GC-MS, ESI-MS and Q-TOF MS/MS to identify the biosurfactants from *Enterobacter* sp. N18. They reported the presence of surfactin homologues such as n-C12, iso-C13, anteiso-C13, iso-C14, n-C14, iso-C15, anteiso-C15, iso-C16, n-C16 and iso-C17 b-OH fatty acids. Korenblum *et al*. (2012) identified surfactin analogues as biosurfactants produced by the *Bacillus* sp. H2O-1 using TLC, GC-MS, ESI-MS and Tandem-MS. These compounds contained a similar heptapeptide chain of surfactin (Glu-Leu-Leu-Val-Asp-Leu-Leu) as the hydrophilic moiety, and C13, C14, C15, C16 β-hydroxy-fatty acids as the hydrophobic moiety. TLC, GC-MS and MALDI TOF-MS/MS was used to identify biosurfactants derived from *P. fluorescens* BD5 (Janek *et al*., 2010). The authors reported two cyclic peptides namely pseudofactin I (palmitoyl-Gly–Ser–Thr–Leu–Leu–Ser–Leu–Val-O-) and pseudofactin II (palmitoyl-Gly–Ser–Thr–Leu–Leu–Ser–Leu–Leu-O-).

The biosurfactants from *B. subtilis* 309, *B. subtilis* 311 and *B. subtilis* 573 were identified by Pereira *et al*. (2013) using FTIR-ATR, $^{1}$H NMR and MALDI-TOF MS/MS. They found that the biosurfactants were made of a heptapeptide (L-Glu-L-Leu-D-Leu-L-Val-L-Asp-D-Leu-L-Leu) linked to a C13, C14 or C15 hydroxy fatty acid chain. Lichenysin–A, is a compound made of a small peptide (Gln, Leu, Leu, Val, Asp, Leu, and Ile) that was linked to 3-hydroxy fatty acid residue (3-hydroxylated tri, tetra, penta, or hexadecanoic acids) with amide and lactone bonds forming a cyclic structure, was identified as the biosurfactants produced by *Bacillus licheniformis* W16 while using FTIR, HPTLC–ESI–MS and MALDI-TOF-MS, $^{1}$H and $^{13}$C NMR as analytical techniques. In the study conducted by Oluwaseun *et al.* (2017), L-rhamnosyl-L-rhamnosyl-3-b-hydroxydodecenoate was identified as the biosurfactants from *Pseudomonas aeruginosa* C1501 using LC-ESI-MS and $^{1}$H and $^{13}$C NMR.

Saravanakumari and Mani (2010) used GC-MS and $^{1}$H NMR to identify O-methyl-β-D-xylopyranoside and octadecanoic acid was the major constitutive elements of the biosurfactants from *L. lactis*. The final structure proposed by the authors was 2-methyl-O-methyl-β-D-xylopyranosyl octadecanoic acid. The combination of TLC, GC-MS, ESI-MS and NMR ($^1$H and $^{13}$C) at one and two dimensions (COSY, HMBC and HSQC) was used in the characterization process of biosurfactants from *L. plantarum* (Sauvageau *et al.*, 2012). The authors identified four fractions including α-D-Glucopyranosyl-diglyceride (GL1), α-D-Galactopyranosyl-(1→2)-α-D-Glucopyranosyl-diglyceride (GL2a), β-D-Glucopyranosyl-(1→6)-β-D-Galactopyranosyl-(1→2)-6-O-acyl-α-D-Glucopyranosyl-diglyceride (GL2b) and β-DGlucopyranosyl-(1→6)-α-D-Galactopyranosyl-(1→2)-α-D-Glucopyranosyl-diglyceride (GL3). Xylopyranosyl linked to octadecanoic acid was identified as the biosurfactants from *L. helveticus* MRTL91 while using TLC, UPLC-ESI-MS, GC-MS, FTIR and NMR ($^1$H and $^{13}$C) (Sharma *et al.*, 2014). Using the same techniques latter, the authors identified xylopyranosyl β-hydroxydecanoic acid as the biosurfactants produced by *E. faecium* MRTL9 (Sharma *et al.*, 2015). Mouafo *et al*. (2020) identified 2,5-O-methyl-rhamnofuranosyl-palmitate in biosurfactants from *L. casei* TM1B using elemental analysis, FTIR, GC-MS, and NMR ($^1$H and $^{13}$C).

## Conclusion

This review presents the different techniques of purification of biosurfactants. It suggests that, the choice of the purification techniques of biosurfactants depends on the producing strain, the culture media composition and the extraction method. Chromatographic, spectrometric and mass spectrometry methods are suitable for fully characterization at structural level of biosurfactants. However, biosurfactants which are known as complex mixture of compounds must be fractionated before being analyzed. They are in the food industry to enhance the shelf live of food, remove biofilms from food processing surfaces, stabilize emulsions, improve dough stability and texture of bakery products. In agricultural field, they are used as biopesticides. In environmental field, they are used for bioremediation, to enhance oil recovery and remove heavy metals from contaminated soils. They are also used in cosmetic, detergency and painting. The full structural characterization of biosurfactants will improve their added values and their application in some highly pointed industrial fields such as medicine and pharmacy.

## Data Availability Statement:

Upon request, the data will be made available from the corresponding author.

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## List of Tables

**Table 1.** Purification methods and nature of biosurfactants produced by some microorganisms

| Microorganisms | Method of purification | Nature of biosurfactants | References |
|---|---|---|---|
| *L. casei* subsp. casei TM1B | - Centrifugation (13,000×g, 4°C, 20 min)<br>- Filtration (0.22 µm)<br>- RP-HPLC | Glycolipid | (Mouafo *et al*., 2020) |
| *L. plantarum* CFR 2194 | - Centrifugation (10,000×g, 4 °C, 15 min)<br>- Precipitation (pH 2) | Glycoprotein | (Nelson *et al*., 2020) |
| *B. velezensis* KLP2016 | - TLC<br>- Size exclusion chromatography<br>- Ion exchange chromatography | Lipopeptide | (Dabaghi *et al*., 2023) |
| *B. oceanisediminis* H2 | - TLC<br>- Column chromatography | Lipopeptide | (Meena *et al*., 2021) |
| *B. subtilis* LSFM-05 | - Column chromatography | Lipopeptide | (Satpute et al., 2010) |
| *S. thermophilus* A | - Filtration (0.22 µm)<br>- Dialysis (6000–8000 Da)<br>- Hydrophobic interaction chromatography | Glycolipid | (Sauvageau *et al*., 2012) |
| *L. plantarum* IRL-560 | - TLC<br>- Column chromatography<br>- Size exclusion chromatography | Glycolipid | (Banerjee and Ghosh, 2021) |
| *L. delbrueckii* | - Column chromatography<br>- Dialysis (6000–8000 Da) | Glycolipid | (Sharma and Saharan, 2016) |
| *L. helveticus* MRTL91 | - Dialysis (6000–8000 Da)<br>- Column chromatography | Glycolipid | (de Faria *et al*., 2021) |
| *L. lactis* | - Precipitation (pH 2)<br>- Centrifugation (6000×g, 15 min, 4°C) | Glycolipid | (Sharma *et al*., 2015) |
| *L. rhamnosus* PTCC 1637 | - Filtration (0.22 µm)<br>- Dialysis (6000–8000 Da) | Phosphoglycoprotein | (Satpute *et al*., 2008) |
| *L. paracasei* ssp. *paracasei* A20 | - Filtration (0.22 µm)<br>- Dialysis (6000–8000 Da) | Glycolipoprotein | (Ghasemi *et al*., 2018) |
| *L. acidophilus* NCIM 2903 | - Filtration (0.22 µm)<br>- Dialysis (6000–8000 Da) | Glycolipoprotein | (Vecino *et al*., 2017) |

## List of Figures

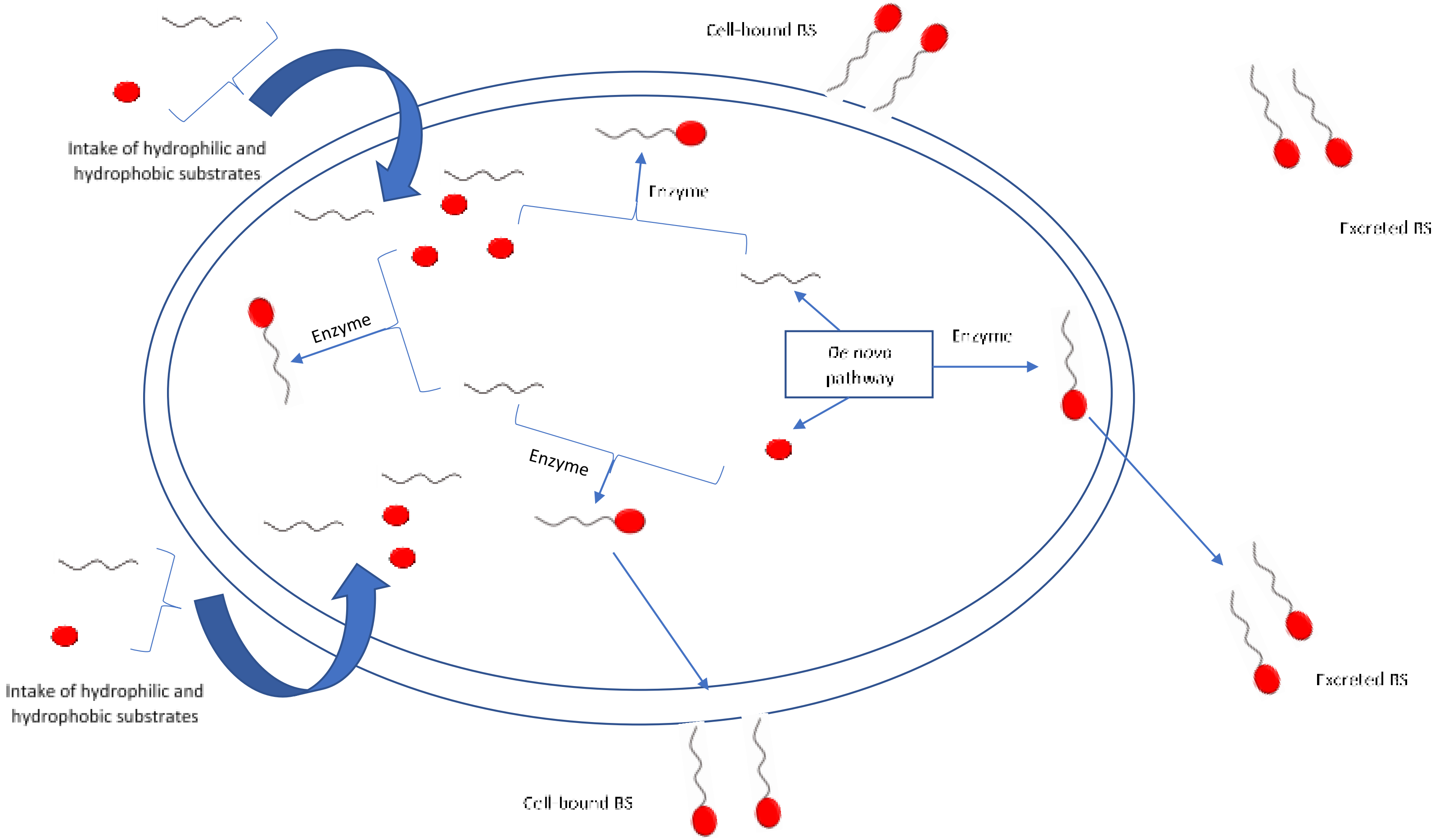

**Fig. 1.** The different metabolism routes of biosurfactants by microorganisms.

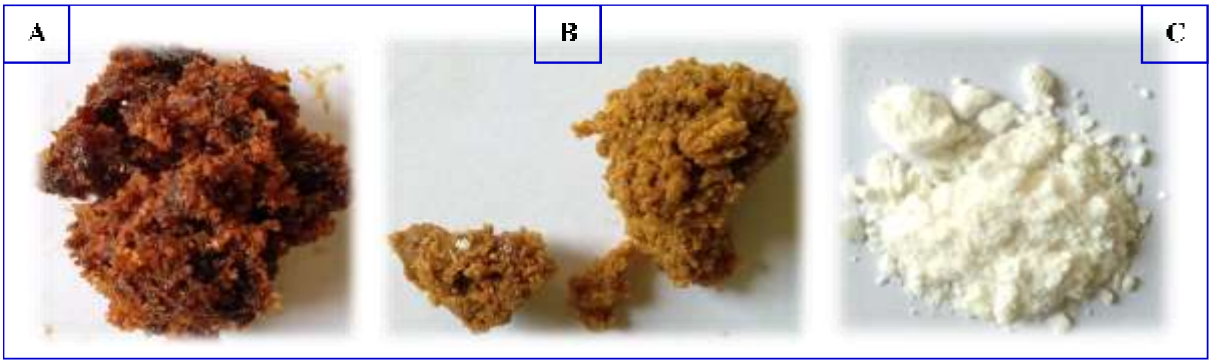

**Fig. 2.** Pictures showing color of biosurfactants produced by *Lactobacillus paracasei* subsp. tolerans N2 with sugar cane molasses as substrate (A), *Lactobacillus casei* subsp. casei TM1B with sugar cane molasses as substrate (B) and *Lactobacillus rhamnosus* G88 with glycerol as substrate (C) (Mouafo, 2019).