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Methods of Purification and Characterization of Biosurfactants: An Overview

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Authors' contributions

This work was carried out in collaboration among all authors. Authors AM and HTM designed and conceived the. Author HTM managed the literature search, performed the methodology, investigation, data curation, formal analysis, and wrote the original draft of the paper. Authors MMP, AJMM, and ATS reviewed and edited the paper. Authors DS and AM provided resources and supervised the study. All authors read and approved the final manuscript.

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Review Article

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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.

1. INTRODUCTION

Biosurfactants are amphiphilic surface-active compounds produced by microorganisms from diverse sources (terrestrial, marine environments, sludge, etc.) including bacteria, molds and yeasts [1,2]. They are either excreted extracellularly or remain bound to the producing cells [3]. They are synthetized by microorganisms through different metabolism routes depending of the nature and the composition of the substrates (Fig. 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 [4]. 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 [5]. According to Desai and Banat [6], 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) [2,7,8]. Considering the molecular weight, biosurfactants are grouped as low molecular weight (glycolipids, lipopeptides, phospholipids) and high molecular weight (particular biosurfactants, polymeric biosurfactants) compounds [9].

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 [8,10,11,12]. 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 [2,8,13,14,15,16,17]. As examples, Wattoo et al. [18] highlighted the application of biosurfactants as emulsifiers and antimicrobial agents in the stabilization and preservation of juices. Reshmy et al. [19] reported the exploitation of the antioxidant properties of biosurfactants in reducing lipids oxidation and thus, improving the preservation of lipids-containing foods. The improvement in the shelf life of raw ground goat meat using biosurfactants was reported by Mouafo et al. [20]. The authors noticed a significant inhibition of microbial proliferation, lipids oxidation, proteins degradation, and color changes. Fookao et al. [21] demonstrated the ability of biosurfactants to improve the texture and dough stability of milk bread. The beneficial effects of biosurfactants in enhancing the texture profile [22,23] and sensory attributes [24] of starchy foods were recently noticed. Numerous other recent applications of the different properties of biosurfactants in the food industry were summarized in the book of Inamuddin and Adetunji [25]. In view of improving the oral bioavailability of hydrophobic drugs at the target sites, the emulsification properties of biosurfactants were successfully exploited as drug delivery system [26,27,28]. For different administration routes of drugs (intravenous, oral, ocular, nasal and topical), there is nowadays the formulation of microemulsion drug delivery systems with biosurfactants that insures their delivery to targets sites and significantly enhance their bioavailability [27]. Besides, there are also interesting reports highlighting the successful applications of biosurfactants for the bioremediation of heavy metals (Cd, Cu, and Pb) in contaminated soils [29]. All these industrial exploitations of biosurfactants justify the increasing demand of biosurfactants for which the markets reached 2210.5 million dollars by 2018 [30] and the high number of companies (approximately 17) around the world, which currently produced biosurfactants [31,32].

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 [33,34]. 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) [35,36,37]. For the other microorganisms, almost researches performed on biosurfactants emphasized their functional groups characterization [1,2,3,38,39,40]. 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 [2]. 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".

1.1 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.

1.1.1 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. [42] 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 [55] 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.

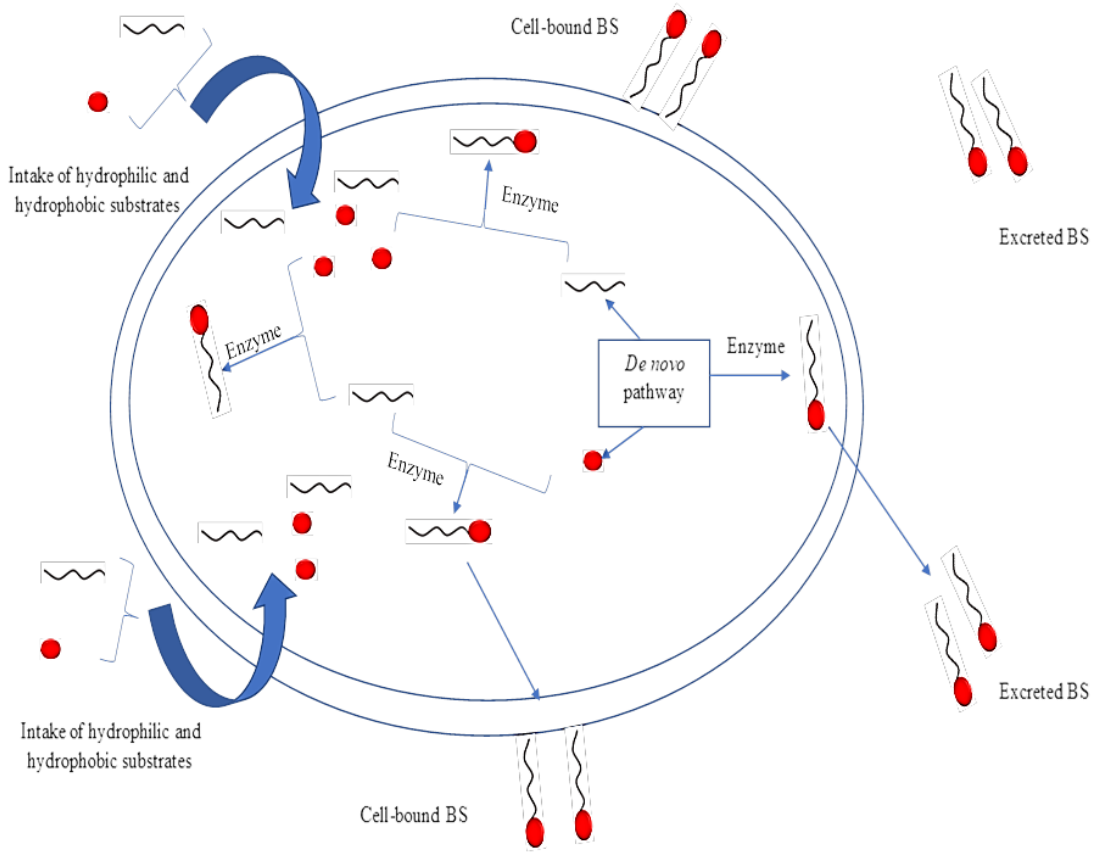

Fig. 1. The different metabolism routes of biosurfactants by microorganisms

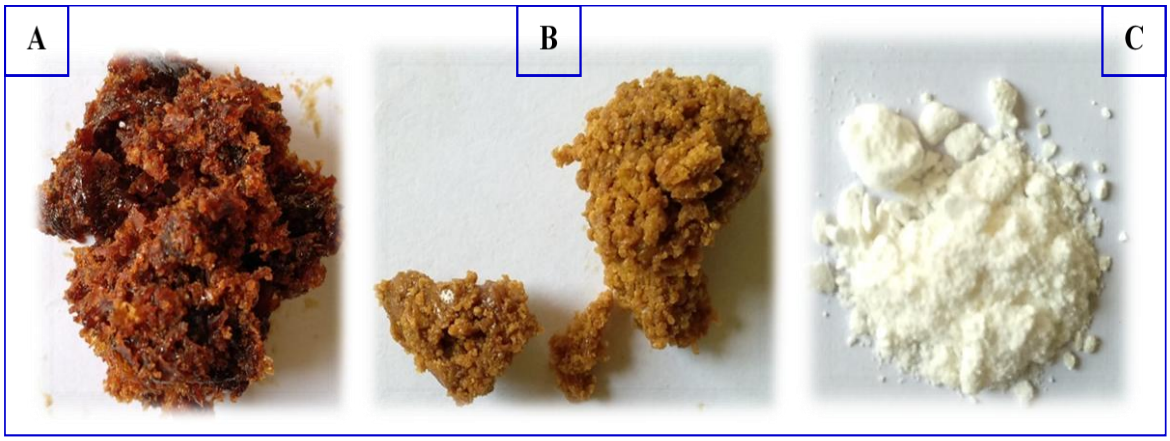

Fig. 2. Pictures showing color of biosurfactants produced by *Lactobacillus paracasei* subsp. tolerans N2 with sugar cane molasses as substrate (A), *Lactobacillus casei* subsp. casei TM1B with sugar cane molasses as substrate (B) and *Lactobacillus rhamnosus* G88 with glycerol as substrate (C) [41]

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) - Filtration (0.22 µm) - RP-HPLC	Glycolipid	[42]
L. plantarum CFR 2194	- Centrifugation (10,000×g, 4 °C, 15 min) - Precipitation (pH 2)	Glycoprotein	[43]
B. velezensis KLP2016	- TLC - Size exclusion chromatography - Ion exchange chromatography	Lipopeptide	[44]
B. oceanisediminis H2	- TLC - Column chromatography	Lipopeptide	[45]
B. subtilis LSFM-05	- Column chromatography	Lipopeptide	[46]
S. thermophilus A	- Filtration (0.22 µm) - Dialysis (6000–8000 Da) - Hydrophobic interaction chromatography	Glycolipid	[47]
L. plantarum IRL-560	- TLC - Column chromatography - Size exclusion chromatography	Glycolipid	[48]
L. delbrueckii	- Column chromatography - Dialysis (6000–8000 Da)	Glycolipid	[49]
L. helveticus MRTL91	- Dialysis (6000–8000 Da) - Column chromatography	Glycolipid	[50]
L. lactis	- Precipitation (pH 2) - Centrifugation (6000×g, 15 min, 4°C)	Glycolipid	[51]
L. rhamnosus PTCC 1637	- Filtration (0.22 µm) - Dialysis (6000–8000 Da)	Phosphoglycoprotein	[52]
L. paracasei ssp. *paracasei* A20	- Filtration (0.22 µm) - Dialysis (6000–8000 Da)	Glycolipoprotein	[53]
L. acidophilus NCIM 2903	- Filtration (0.22 µm) - Dialysis (6000–8000 Da)	Glycolipoprotein	[54]

1.1.2 Precipitation

Almost biosurfactants are known for their low solubility under acidic conditions [43]. That property was exploited by several authors to purify biosurfactants from other contaminants. Madhu and Paprulla [55] 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 [56], chilled ethanol [21,57] and ammonium sulphate [58].

1.1.3 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 [42,47,59].

1.1.3.1 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 [44,45,47,48,60].

1.1.3.2 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. [59] 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. [61] and Sharma and Saharan [49] 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 [42]. A silica gel column (60 Mesh) with chloroform–methanol-water (65:25:4) as mobile phase was successfully used by Banerjee and Ghosh [48] 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 [45]. 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 [50]. Vigneshwaran et al. [58] 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 [49,61]. This might reduce the probability to discovery new biosurfactants.

1.1.4 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 [46]. 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 [42,62]. It is important to highlight that, filtration is always used as a unit operation included in a purification process that contains other purification techniques like TLC, column chromatography, etc.

1.1.5 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. [54], Ghasemi et al. [53], Satpute et al. [62] and Vigneshwaran et al. [58] 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 [2].

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 [52]. 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 [52]. This method was successfully used to confirm the anionic nature of biosurfactants derived from *Lactococcus lactis* [63], *E. faecium* [51], and *Pseudomonas guguanensis* strain Iraqi ZG.K.M [64]. 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 [64]. Fig. 2 presents the color of biosurfactants produced by some lactobacilli strains [41].

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 [48,64].

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 [46]. That method was applied directly on a solution of pure biosurfactants by Atta et al. [65]. 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. [66] reported that the TLC plates can be sprayed with anthrone reagent for detection of sugars while Satpute et al. [62] noticed that sugars detection can be assessed through TLC plates spraying with diphenylamine. In the studies of Reddy et al. [67], 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 [64,66]. The method is not specific as a positive result might indicate the presence of neutral or polar lipids [65]. According to de Faria et al. [50] and Meena et al. [45], 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 [59]. A rapid method to assess phospholipids in biosurfactants was developed by Okpokwasili and Ibiene [68]. 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 [69] was used by Ghasemi et al. [70] and Behzadnia et al. [71] 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 [72]. In the study of Morais et al. [73], the method of Lowry et al. [74] 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 [75] was used by Mouafo et al. [76] 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. [77] by Ferreira et al. [78] and Ghasemi et al. [70]. 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) [2], 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 [73].

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. [79]. 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. [78], Mouafo et al. [12], Mouafo et al. [76], Behzadnia et al. [71], Mouafo et al. [42] and Devale et al. [57] 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. [59], 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. [80] to assess the elemental composition of biosurfactants from *P. benzoelyticum* Pb4, *Bacillus albus* S2i and *Proteus mirabilis* Th1. Ferreira et al. [78], and Vecino et al. [54] 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. [76] 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 [81]. 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 [42,44,48,54,64,73,76,80]. 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 [40,82]. The ultraviolet spectroscopy method was used by Dehghan-Noudeh et al. [83] and Ismail et al. [84] 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. [40] 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 [72]. 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 [17,42,51,54,64,71,73,85].

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. [47], Morais et al. [73], Mouafo et al. [42] and Mouafo et al. [17] 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) [35]. 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. [86] 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 [9]. 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 [9]. 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 [87].

Opposite to the Edman degradation technique, Q-TOF MS/MS is applied directly on the entire biosurfactants without a preliminary hydrolysis step [86]. 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. [88]. 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 [9]. Korenblum et al. [89] 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 [90]. The same method was used by Pereira et al. [91] 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. [92] 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 [72]. 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 [93]. Fouad et al. [94] 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. [62] using SDS-PAGE. Tricine-SDS-PAGE was performed by Vigneshwaran et al. [58] 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 [17,37,85,92,95,96,97].

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 [98]. 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 [99].

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 [17,42,44,47,51,91,96,100]. 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 [47].

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. [43] 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. [95] 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. [86] 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. [89] 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 [90]. 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. [91] 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. [96], 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 [63] 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* [47]. 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) [101]. Using the same techniques latter, the authors identified xylopyranosyl β-hydroxydecanoic acid as the biosurfactants produced by *E. faecium* MRTL9 [51]. Mouafo et al. [42] 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).

8. 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 life 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.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Nataraj BH, Ramesh C, Mallappa RH. Characterization of biosurfactants derived from probiotic lactic acid bacteria against methicillin-resistant and sensitive *Staphylococcus aureus* isolates. LWT Food Sci Technol. 2021a;151:112195. DOI: 10.1016/j.lwt.2021.112195.
2. Mouafo HT, Sokamte AT, Mbawala A, Ndjouenkeu R, Devappa S. Biosurfactants from lactic acid bacteria: a critical review on production, extraction, structural characterization and food application. Food Biosci. 2022;46:101598. DOI: 10.1016/j.fbio.2022.101598.
3. Vallejo CM, Restrepo MAF, Duque FLG, Díaz JCQ. Production, characterization and kinetic model of biosurfactant produced by lactic acid bacteria. Elec. J Biotechnol. 2021. DOI: 10.1016/j.ejbt.2021.06.001.
4. Tokumoto Y, Nomura N, Uchiyama H, Imura T, Morita T, Fukuoka T et al. Structural characterization and surface-active properties of a succinoyl trehalose lipid produced by *Rhodococcus* sp. SD-74. J Oleo Sci. 2009;58(2):97-102. DOI: 10.5650/jos.58.97
5. Syldatk C, Wagner F. Production of biosurfactants. In *Biosurfactants and Biotechnology*; Kosaric N, Cairns WL, Gray NCC, Eds.; Marcel Dekker: New York, NY, USA. 1987;89–120.
6. Desai JD, Banat IM. Microbial production of surfactants and their commercial potential. Microbiol Mol Biol Rev. 1997; 61(1):47-64. DOI: 10.1128/mmbr.61.1.47-64.1997
7. Bergevin M, Marion A, Farber D, Golding GR, Lévesque S. Severe MRSA enterocolitis caused by a strain harboring enterotoxins DG, and I. Emerg Infect Dis. 2017;23(5):865-7.
8. Nitschke MN, Silva SSE. Recent food applications of microbial surfactants. Crit Rev Food Sci Nutr. 2018;58(4):631-8. DOI: 10.1080/10408398.2016.1208635
9. Smyth TJ, Rudden M, Tsaousi K, Marchant R, Banat IM. Protocols for the isolation and analysis of lipopeptides and bioemulsifiers. *In* T.J. McGenity et al. (eds.), Hydrocarbon and lipid microbiology protocols, Springer Protocols Handbooks, Springer-Verlag Berlin Heidelberg. 2014. Available:https://doi.org/10.1007/8623_2014_29.
10. Marchant R, Banat IM. Microbial biosurfactants: challenges and opportunities for future exploitation. Trends Biotechnol. 2012a;30(11):558-65. DOI: 10.1016/j.tibtech.2012.07.003
11. Marchant R, Banat IM. Biosurfactants: a sustainable replacement for chemical surfactants? Biotechnol Lett. 2012b;34(9): 1597-605. DOI: 10.1007/s10529-012-0956-x
12. Mouafo TH, Mbawala A, Ndjouenkeu R. Effect of different carbon sources on biosurfactants' production by three strains of *Lactobacillus* spp. BioMed Res Int. 2018a;2018:5034783. DOI: 10.1155/2018/5034783
13. Silva IA, Veras BO, Ribeiro BG, Aguiar JS, Campos Guerra JMC, Luna JM et al. Production of cupcake-like dessert containing microbial biosurfactant as an emulsifier. PeerJ. 2020;8:e9064. DOI: 10.7717/peerj.9064
14. Patel M, Siddiqui AJ, Hamadou WS, Surti M, Awadelkareem AM, Ashraf SA et al. Inhibition of bacterial adhesion and antibiofilm activities of a glycolipid biosurfactant from *Lactobacillus rhamnosus* with Its physicochemical and functional properties. Antibiotics (Basel). 2021;10(12):1546. DOI: 10.3390/antibiotics10121546
15. Mouafo TH, Somashekar D. Biosurfactants and its application in the food industry. Indian Food Ind Mag. 2020;4(1):49-57.

16. Mouafo HT, Baomog AMB, Adjele JJB, Sokamte AT, Mbawala A, Ndjouenkeu R. Microbial profile of fresh beef sold in the markets of Ngaoundéré, Cameroon, and antiadhesive activity of biosurfactants against selected bacterial pathogens. J Food Qual. 2020c;2020:1-10.
DOI: 10.1155/2020/5989428.
17. Mouafo HT, Sokamte AT, Manet L, Mbarga AJM, Nadezdha S, Devappa S et al. Biofilm inhibition, antibacterial and antiadhesive properties of a novel biosurfactant from *Lactobacillus paracasei* N2 against multi-antibiotics-resistant pathogens isolated from braised fish. Fermentation. 2023;9(7):646.
DOI: 10.3390/fermentation9070646.
18. Wattoo AG, Yaseen S, Kanwal M, Zaidi SMM, Alamgeer M, Saeed D et al. Application of biosurfactants in juice industry. In: Inamuddin, Adetunji CO, editors. Applications of next generation biosurfactants in the food sector. Academic Press, Elsevier Inc; 2023. p. 527-36.
DOI: 10.1016/B978-0-12-824283-4.00013-7.
19. Reshmy R, Philip E, Madhavan A, Binod P, Awasthi MK, Pandey A, Sindhu R. Applications of biosurfactant as an antioxidant in foods. *In* Inamuddin and Adetunji C.O. 2023. Applications of Next Generation Biosurfactants in the Food Sector. Academic Press, Elsevier Inc., 2023:391-401.
Available: https://doi.org/10.1016/B978-0-12-824283-4.00008-3.
20. Mouafo HT, Mbawala A, Tanaji K, Somashekar D, Ndjouenkeu R. Improvement of the shelf life of raw ground goat meat by using biosurfactants produced by lactobacilli strains as biopreservatives. LWT Food Sci Technol. 2020b;133:110071.
DOI: 10.1016/j.lwt.2020.110071.
21. Fookao AN, Mbawala A, Nganou ND, Nguimbou RM, Mouafo HT. Improvement of the texture and dough stability of milk bread using bioemulsifiers/biosurfactants produced by lactobacilli isolated from indigenous fermented milk (*pendidam*). LWT Food Sci Technol. 2022;163:113609.
DOI: 10.1016/j.lwt.2022.113609.
22. Santoso SP, Angkawijaya AE, Kurniawan A, Cheng KC, Hsieh CW. The role of biosurfactants in the improvement of texture and shelf life of starch-containing products. *In* Inamuddin and Adetunji C.O. Applications of Next Generation Biosurfactants in the Food Sector. Academic Press, Elsevier Inc., 2023;149-169.
Available: https://doi.org/10.1016/B978-0-12-824283-4.00018-6.
23. Sharma A, Arya SK. The application of biosurfactant for the modification of the rheological properties of wheat dough. *In* Inamuddin and Adetunji C.O. Applications of Next Generation Biosurfactants in the Food Sector. Academic Press, Elsevier Inc., 2023;171-186.
Available: https://doi.org/10.1016/B978-0-12-824283-4.00016-2.
24. Rahaman SM, Bhattarai A, Kumar D, Singh B, Saha B. Application of biosurfactants as emulsifiers in the processing of food products with diverse utilization in the baked goods. *In* Inamuddin and Adetunji C.O. Applications of Next Generation Biosurfactants in the Food Sector. Academic Press, Elsevier Inc., 2023;203-237.
Available: https://doi.org/10.1016/B978-0-12-824283-4.00021-6.
25. Inamuddin and Adetunji CO. Applications of Next Generation Biosurfactants in the Food Sector. Academic Press, Elsevier Inc. 2023;545.
Available:https://doi.org/10.1016/C2019-0-05486-6.
26. Kurozuka A, Onishi S, Nagano T, Yamaguchi K, Suzuki T, Minami H. Emulsion polymerization with a biosurfactant. Langmuir. 2017;33(23): 5814-8.
DOI: 10.1021/acs.langmuir.7b00851
27. Ohadi M, Shahravan A, Dehghannoudeh N, Eslaminejad T, Banat IM, Dehghannoudeh G. Potential use of microbial surfactant in microemulsion drug delivery system: a systematic review. Drug Des Dev Ther. 2020;14:541-50.
DOI: 10.2147/DDDT.S232325
28. Bjerk TR, Severino P, Jain S, Marques C, Silva AM, Pashirova T et al. Biosurfactants: properties and applications in drug delivery, biotechnology and ecotoxicology. Bioengineering (Basel). 2021;8(8):115.
DOI: 10.3390/bioengineering8080115
29. Sun W, Zhu B, Yang F, Dai M, Sehar S, Peng C et al. Optimization of biosurfactant production from *Pseudomonas* sp. CQ2 and its application for remediation of heavy

metal contaminated soil. Chemosphere. 2021;265:129090.
DOI: 10.1016/j.chemosphere.2020.129090

30. Transparency Market Research. Specialty surfactants market and biosurfactants market: Global scenario, raw material and consumption trends, industry analysis, size, share and forecast. 2010–2018. 2013. Available from http://www.transparencymarketresearch.com/specialty-and-biosurfactants-market.html. Accessed November 15, 2015.
31. Randhawa KKS, Rahman PKSM. Rhamnolipid biosurfactants – past, present, and future scenario of global market. Front Microbiol. 2014;5:454.
32. Sekhon Randhawa KK, Rahman PK. Rhamnolipid biosurfactants–past, present and future scenario of global market. Front Microbiol. 2014;5:454.
DOI: 10.3389/fmicb.2014.00454
33. Mulligan CN, Sharma SK, Mudhoo A. Biosurfactants research trends and applications. Boca Raton, London, New-York: Taylor & Francis Group, LLC. 2014;346.
34. Liu JF, Mbadinga SM, Yang SZ, Gu JD, Mu BZ. Chemical structure, property and potential applications of biosurfactants produced by *Bacillus subtilis* in petroleum recovery and spill mitigation. Int J Mol Sci. 2015;16(3):4814-37.
35. Joshi SJ, Al-wahaibi YM, Al-bahry SN, Elshafie AE, Ennis CJ. Production, characterization, and application of *Bacillus licheniformis* W16 biosurfactant in enhancing oil recovery. Front Microbiol. 2016;7:1-14.
DOI: 10.3389/fmicb.2016.01853.
36. Sharma D, Saharan BS, Kapil S. Biosurfactants of lactic acid bacteria. Springer briefs in microbiology. Springer international publishing AG Switzerland; 2016.
DOI: 10.1007/978-3-319-26215-4.
37. Abdalsadiq N, Hassan Z, Lani M, Bulgasem B. Identification of biosurfactant produced by *Lactobacillus* spp. using mass spectrometry. Res J Life Sci Bioinformatics Pharm Chem Sci. 2018;4(4):247-58.
DOI: 10.26479/2018.0404.21.
38. Lara VM, Vallejo M, Parada R, Ossa JSH, Gliemmo MF, Campos CA. Characterization of the emulsifying activity of biosurfactants produced by lactic acid bacteria isolated from the Argentinian Patagonia. J Disp Sci Technol. 2020:1-8.
DOI: 10.1080/01932691.2020.1845961.
39. Nataraj BH, Ramesh C, Mallappa RH. Functional group characterization of lactic bacterial biosurfactants and evaluation of antagonistic actions against clinical isolates of methicillin-Resistant *Staphylococcus aureus* (MRSA). Lett Appl Microbiol. 2021b;73(3):372-82.
DOI: 10.1111/lam.13523
40. Sakr EAE, Ahmed HAE, Abo Saif FAAA. Characterization of low-cost glycolipoprotein biosurfactant produced by *Lactobacillus plantarum* 60 FHE isolated from cheese samples using food wastes through response surface methodology and its potential as antimicrobial, antiviral, and anticancer activities. Int J Biol Macromol. 2021;170:94-106.
DOI: 10.1016/j.ijbiomac.2020.12.140
41. Mouafo TH. Production of biosurfactants by *Lactobacillus* spp. strains using sugar cane molasses as substrate: Application to the biopreservation of fresh ground beef. Ph.D thesis. National School of Agro-Industrial Sciences (ENSAI), University of Ngaoundéré, Cameroon; 2019.
42. Mouafo TH, Mbawala A, Somashekar D, Tchougang HM, Harohally NV, Ndjouenkeu R. Biological properties and structural characterization of a novel rhamnolipid like-biosurfactants produced by *Lactobacillus casei* subsp. casei TM1B. Biotechnol Appl Biochem. 2020a:1-12.
DOI: 10.1002/bab.1966.
43. Nelson J, El-Gendy AO, Mansy MS, Ramadan MA, Aziz RK. The biosurfactants iturin, lichenysin and surfactin, from vaginally isolated lactobacilli, prevent biofilm formation by pathogenic *Candida*. FEMS Microbiol Lett. 2020;367(15):1-9.
DOI: 10.1093/femsle/fnaa126
44. Dabaghi S, Ataei SA, Taheri A. Production of rhamnolipid biosurfactants in solid-state fermentation: process optimization and characterization studies. BMC Biotechnol. 2023;23(1):2.
DOI: 10.1186/s12896-022-00772-4
45. Meena KR, Dhiman R, Singh K, Kumar S, Sharma A, Kanwar SS et al. Purification and identification of a surfactin biosurfactnat and engine oil degradation by *Bacillus velezensis* KLP2016. Microb Cell Factories. 2021;20(26):1-12.
DOI: 10.1186/s12934-021-01519-0.

46. Satpute SK, Banpurkar AG, Dhakephalkar PK, Banat IM, Chopade BA. Methods for investigating biosurfactants and bioemulsifiers: a review. Crit Rev Biotechnol. 2010;30(2):127-44.
DOI: 10.3109/07388550903427280
47. Sauvageau J, Ryan J, Lagutin K, Sims IM, Stocker BL, Timmer MSM. Isolation and structural characterisation of the major glycolipids from *Lactobacillus plantarum*. Carbohydr Res. 2012;357:151-6.
DOI: 10.1016/j.carres.2012.05.011
48. Banerjee S, Ghosh U. Production, purification and characterization of biosurfactant isolated from *Bacillus oceanisediminis* H2. Mater Today Proc. 2023;81:1012-6.
DOI: 10.1016/j.matpr.2021.04.366.
49. Sharma D, Saharan BS. Functional characterization of biomedical potential of biosurfactant produced by *Lactobacillus helveticus*. Biotechnol Rep (Amst). 2016;11:27-35.
DOI: 10.1016/j.btre.2016.05.001
50. de Faria AF, Stéfani D, Vaz BG, Silva ÍS, Garcia JS, Eberlin MN et al. Purification and structural characterization of fengycin homologues produced by *Bacillus subtilis* LSFM-05 grown on raw glycerol. J Ind Microbiol Biotechnol. 2011;38(7):863-71.
DOI: 10.1007/s10295-011-0980-1
51. Sharma D, Saharan BS, Chauhan N, Procha S, Lal S. Isolation and functional characterization of novel biosurfactant produced by *Enterococcus faecium*. SpringerPlus. 2015;4(1):4.
DOI: 10.1186/2193-1801-4-4
52. Satpute SK, Bhawsar BD, Dhakephalkar PK, Chopade BA. Assessment of different screening methods for selecting biosurfactant producing marine bacteria. Indian J Mar Sci. 2008;37(3):243-50.
53. Ghasemi A, Moosavi-Nasab M, Behzadnia A, Rezaei M. Enhanced biosurfactant production with low-quality date syrup by *Lactobacillus rhamnosus* using a fed-batch fermentation. Food Sci Biotechnol. 2018;27(4):1137-44.
DOI: 10.1007/s10068-018-0366-5
54. Vecino X, Rodríguez-López L, Gudiña EJ, Cruz JM, Moldes AB, Rodrigues LR. Vineyard pruning waste as an alternative carbon source to produce novel biosurfactants by *Lactobacillus paracasei*. J Ind Eng Chem. 2017;55:40-9.
DOI: 10.1016/j.jiec.2017.06.014.
55. Madhu AN, Paprulla SG. Evaluation and functional characterization of a biosurfactant Produced by *Lactobacillus plantarum* CFR 2194. Appl Biochem Biotechnol. 2013:1-13.
DOI: 10.1007/s12010-013-0649-5.
56. Bhosale SS, Rohiwal SS, Chaudhary LS, Pawar KD, Patil PS, Tiwari AP. Photocatalytic decolorization of methyl violet dye using rhamnolipid biosurfactant modified iron oxide nanoparticles for wastewater treatment. J Mater Sci: Mater Electron. 2019;30(5):4590-8.
DOI: 10.1007/s10854-019-00751-0.
57. Devale A, Sawant R, Pardesi K, Perveen K, Khanam MN, Shouche Y et al. Production and characterization of bioemulsifier by *Parapedobacter indicus*. Front Microbiol. 2023;14:1111135.
DOI: 10.3389/fmicb.2023.1111135
58. Vigneshwaran C, Vasantharaj K, Krishnanand N, Sivasubramanian V. Production Optimization, Purification and characterization of lipopeptide biosurfactant obtained from *Brevibacillus* sp. AVN13. J Environ Chem Eng. 2021;9(1):104867.
DOI: 10.1016/j.jece.2020.104867.
59. Rodrigues LR, Teixeira JA, van der Mei HC, Oliveira R. Isolation and partial characterization of a biosurfactant produced by *Streptococcus thermophilus* A. Coll. Colloids Surf B Biointerfaces. 2006;53(1):105-12.
DOI: 10.1016/j.colsurfb.2006.08.009
60. Fernandes NdAT, Simões LA, Dias DR. Biosurfactants produced by yeasts: fermentation, screening, recovery, purification, characterization, and applications. Fermentation. 2023;9(3):207.
DOI: 10.3390/fermentation9030207.
61. Thavasi R, Jayalakshmi S, Banat IM. Application of biosurfactant produced from peanut oil cake by *Lactobacillus delbrueckii* in biodegradation of crude oil. Bioresour Technol. 2011;102(3):3366-72.
DOI: 10.1016/j.biortech.2010.11.071
62. Satpute SK, Mone NS, Das P, Banat IM, Banpurkar AG. Inhibition of pathogenic bacterial biofilms on PDMS based implants by *L. acidophilus* derived biosurfactant. BMC Microbiol. 2019;19(1):39.
DOI: 10.1186/s12866-019-1412-z
63. Saravanakumari P, Mani K. Structural characterization of a novel xylolipid biosurfactant from *Lactococcus lactis* and analysis of antibacterial activity against

multi-drug resistant pathogens. Bioresour Technol. 2010;101(22):8851-4.
DOI: 10.1016/j.biortech.2010.06.104

64. Faisal ZG, Mahdi MS, Alobaidi KH. Optimization and chemical characterization of biosurfactant produced from a novel *Pseudomonas guguanensis* strain Iraqi ZG.K.M. Int J Microbiol. 2023:1-16.
DOI: 10.1155/2023/1571991.
65. Atta HM, Abul-hamd AT, Radwan HG. Production of Destomycine – A antibiotic by *Streptomyces* sp. using rice straw as fermented substrate. Commun Appl Biol Sci. 2009;74:879-97.
66. Sen S, Borah SN, Bora A, Deka S. Production, characterization, and antifungal activity of a biosurfactant produced by *Rhodotorula babjevae* YS3. Microb Cell Factories. 2017;16(95):1-14.
DOI: 10.1186/s12934-017-0711-z
67. Reddy KS, Khan MY, Archana K, Reddy MG, Hameeda B. Utilization of mango kernel oil for the rhamnolipid production by *Pseudomonas aeruginosa* DR1 towards its application as biocontrol agent. Biores Technol. 2016;221:291-9.
DOI: 10.1016/j.biortech.2016.09.041.
68. Okpokwasili GC, Ibiene AA. Enhancement of recovery of residual oil using a biosurfactant slug. Afr J Biotechnol. 2006;5(5):453-6.
69. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72:248-54.
DOI: 10.1006/abio.1976.9999
70. Ghasemi A, Moosavi-nasab M, Setoodeh P, Mesbahi G, Yousefi G. Biosurfactant production by lactic acid bacterium *Pediococcus dextrinicus* SHU1593 grown on different carbon sources: strain screening followed by product characterization. Sci Rep. 2019;9(1):5287.
DOI: 10.1038/s41598-019-41589-0
71. Behzadnia A, Moosavi-nasab M, Tiwari BK, Setoodeh P. *Lactobacillus plantarum*-derived biosurfactant: ultrasound-induced production and characterization. Ultrasonics – Sonochem. Ultrason Sonochem. 2020;65:105037.
DOI: 10.1016/j.ultsonch.2020.105037
72. Smyth TJP, Perfumo A, McClean S, Marchant R, Banat IM. Isolation and Analysis of Lipopeptides and High Molecular Weight Biosurfactants. *In*, Timmis, K.N. (ed), *Handbook of Hydrocarbon and Lipid Microbiology*. Springer-Verlag, Berlin Heidelberg, 2010 ;3687–3704.
Available: https://doi.org/10.1007/978-3-540-77587-4_290.
73. Morais IMC, Cordeiro AL, Teixeira GS, Domingues VS, Nardi RMD, Monteiro AS, Alves RJ, Siqueira EP, Santos VL. Biological and physicochemical properties of biosurfactants produced by *Lactobacillus jensenii* - P 6A and *Lactobacillus gasseri* - P 65. Microbial Cell Fact. 2017:1–15.
Available: https://doi.org/10.1186/s12934-017-0769-7.
74. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol regent. J Biol Chem. 1951;193(1):265-75.
75. AOAC. Official methods of analysis of AOAC International, Method 960.52 (surplus method). 15th ed. Arlington, VA: Association of Analytical Chemists; 1990.
76. Mouafo HT, Mbawala A, Tchougang MH, Ndjouenkeu R, Somashekar D. Application of response surface methodology to improve the production of antimicrobial biosurfactants by *Lactobacillus paracasei* subsp. tolerans N2 using sugar cane molasses as substrate. Biores Bioprocess. 2018b;5(48):1-16.
DOI: 10.1186/s40643-018-0234-4.
77. Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purifcation of total lipides from animal tissues. J Biol Chem. 1957;226(1):497-509.
78. Ferreira A, Vecino X, Ferreira D, Cruz JM, Moldes AB, Rodrigues LR. Novel cosmetic formulations containing a biosurfactant from *Lactobacillus paracasei*. Colloids Surf B Biointerfaces. 2017;155:522-9.
DOI: 10.1016/j.colsurfb.2017.04.026
79. Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. Anal Chem. 1956;28(3):350-6.
DOI: 10.1021/ac60111a017.
80. Habib S, Ahmed A, Haque MFU, Ejaz R. Efficacious use of potential biosurfactant producing plant growth promoting rhizobacteria to combat petrol toxicity in *Zea mays* L. plants. Environ Sci Pollut Res Int. 2023;30(18):53725-40.
DOI: 10.1007/s11356-023-25925-6
81. Jemil N, Ben Ayed H, Manresa A, Nasri M, Hmidet N. Antioxidant properties,

antimicrobial and anti-adhesive activities of DCS1 lipopeptides from *Bacillus methylotrophicus* DCS1. BMC Microbiol. 2017;17(1):144.
DOI: 10.1186/s12866-017-1050-2

82. Wilcox CF, Wilcox MF. Visible and ultraviolet spectroscopy. *In*: Experimental Organic Chemistry, second Ed. Prentice-Hall, New Jersey. 1995;254–262.
83. Dehghan-Noude G, Housaindokht M, Bazzaz BSF. Isolation, characterization, and investigation of surface and hemolytic activities of a lipopeptide biosurfactant produced by *Bacillus subtilis* ATTC 6633. J Microbiol. 2005;43(3):272-6
84. Ismail W, Al-Rowaihi IS, Al-Humam AA, Hamza RY, El Nayal AM, Bououdina M. Characterization of a lipopeptide biosurfactant produced by a crude-oil-emulsifying *Bacillus* sp. I-15. Int Biodeterior Biodegrad. 2013;84:168-78.
DOI: 10.1016/j.ibiod.2012.04.017.
85. De Gregorio PR, Parolin C, Abruzzo A, Luppi B, Protti M, Mercolini L et al. Biosurfactant from vaginal *Lactobacillus crispatus* BC1 as a promising agent to interfere with *Candida* adhesion. Microb Cell Factories. 2020;19(133):1-16.
DOI: 10.1186/s12934-020-01390-5.
86. You J, Yang S, Mu BZ. Structural characterization of lipopeptides from *Enterobacter* sp. strain N18 reveals production of surfactin homologues. Eur J Lipid Sci Technol. 2015;117(6):890-8.
DOI: 10.1002/ejlt.201400386.
87. Zachara NE, Gooley AA. Identification of glycosylation sites in mucin peptides by Edman degradation. Methods Mol Biol. 2000;125:121-8.
DOI: 10.1385/1-59259-048-9:121
88. Yang SZ, Wei DZ, Mu BZ. Determination of the amino acid sequence in a cyclic lipopeptide using MS with DHT mechanism. J Biochem Biophys Methods. 2006;68(1):69-74.
DOI: 10.1016/j.jbbm.2006.03.008
89. Korenblum E, de Araujo LV, Guimarães CR, de Souza LM, Sassaki G, Abreu F et al. Purification and characterization of a surfactin-like molecule produced by *Bacillus* sp. H2O-1 and its antagonistic effect against sulfate reducing bacteria. BMC Microbiol. 2012;12(252):252.
DOI: 10.1186/1471-2180-12-252
90. Janek T, Łukaszewicz M, Rezanka T, Krasowska A. Isolation and characterization of two new lipopeptide biosurfactants produced by *Pseudomonas fluorescens* BD5 isolated from water from the Arctic Archipelago of Svalbard. Bioresour Technol. 2010;101(15):6118-23.
DOI: 10.1016/j.biortech.2010.02.109
91. Pereira JFB, Gudiña EJ, Costa R, Vitorino R, Teixeira JA, Coutinho JAP et al. Optimization and characterization of biosurfactant production by *Bacillus subtilis* isolates towards microbial enhanced oil recovery applications. Fuel. 2013;111:259-68.
DOI: 10.1016/j.fuel.2013.04.040.
92. de Faria AF, Teodoro-Martinez DS, de Oliveira Barbosa GN, Gontijo Vaz B, Serrano Silva Í, Garcia JS et al. Production and structural characterization of surfactin (C14/Leu7) produced by Bacillus subtilis isolate LSFM-05 grown on raw glycerol from the biodiesel industry. Process Biochem. 2011;46(10):1951-7.
DOI: 10.1016/j.procbio.2011.07.001.
93. Toren A, Navon-Venezia S, Ron EZ, Rosenberg E. Emulsifying activities of purified alasan proteins from *Acinetobacter radioresisten* HA53. Appl Environ Microbiol. 2001;67(3):1102-6.
DOI: 10.1128/AEM.67.3.1102-1106.2001.
94. Fouad HK, Khanaqa HH, Munira I. Purification and characterization of surlactin produced by *Lactobacillus acidophilus*. Al-Anbar. Med J. 2010;8(1):34-9.
95. Janek T, Łukaszewicz M, Krasowska A. Identification and characterization of biosurfactants produced by the Arctic bacterium *Pseudomonas putida* BD2. Colloids Surf B Biointerfaces. 2013;110:379-86.
DOI: 10.1016/j.colsurfb.2013.05.008
96. Charles Oluwaseun A, Julius Kola O, Mishra P, Ravinder Singh J, Kumar Singh A, Singh Cameotra S et al. Characterization and optimization of a rhamnolipid from *Pseudomonas aeruginosa* C1501 with novel biosurfactant activities. Sustain Chem Pharm. 2017;6:26-36.
DOI: 10.1016/j.scp.2017.07.001.
97. Adu SA, Twigg MS, Naughton PJ, Marchant R, Banat IM. Characterisation of cytotoxicity and immunomodulatory effects of glycolipid biosurfactants on human keratinocytes. Appl Microbiol Biotechnol. 2023;107(1):137-52.
DOI: 10.1007/s00253-022-12302-5

98. Abdel-Mawgoud AM, Hausmann R, Lepine F, Muller MM, Deziel E. Rhamnolipids: detection, analysis, biosynthesis, genetic regulation, and bioengineering of production. *In* G. Soberon- Chavez (ed.), Biosurfactants, Microbiology Monographs 20, Springer-Verlag Berlin Heidelberg; 2011.
Available: https://doi.org/10.1007/978-3-642-14490-5_2.
99. Hamerly T, Tripet B, Wurch L, Hettich RL, Podar M, Bothner B et al. Characterization of fatty acids in *Crenarchaeota* by GC-MS and NMR. Archaea. 2015;2015:472726.
DOI: 10.1155/2015/472726
100. Monteiro SA, Sassaki GL, de Souza LM, Meira JA, de Araújo JM, Mitchell DA et al. Molecular and structural characterization of the biosurfactant produced by *Pseudomonas aeruginosa* DAUPE 614. Chem Phys Lipids. 2007;147(1):1-13.
DOI: 10.1016/j.chemphyslip.2007.02.001
101. Sharma D, Saharan BS, Chauhan N, Bansal A, Procha S. Production and structural characterization of *Lactobacillus helveticus* derived biosurfactant. Sci World J. 2014;2014:1-9.
DOI: 10.1155/2014/493548.

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