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MICROBIAL BIOSURFACTANTS: METHODS FOR THEIR ISOLATION AND CHARACTERIZATION

Selene García-Reyes[1], *Gustavo Yañez-Ocampo**[2]

Address(es): Dr. Gustavo Yañez-Ocampo
[1]Master in Biotechnology, Biotechnology Research Center, University of the State of Morelos
[2]Laboratory of Edaphology and Environment, Faculty of Sciences, Autonomous University of the State of Mexico, Instituto Literario 100. Toluca Mexico

*Corresponding author: yanezg0206@gmail.com

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ABSTRACT

Biosurfactants are amphiphilic molecules produced by microorganisms. They are exopolymers of low and high molecular weight, capable to emulsify and decrease the surface tension water. Recently, the interest for its production has increased, because of their environmental, industrial, and pharmaceutical applications, and others. However, in order to obtain a biosurfactant, it is necessary to research the producer strain of biosurfactant, its optimal conditions of incubation and carbon sources. Once recovered the biosurfactants, it is required to know their biochemical composition, for this purpose we have developed sensitive and analytical techniques to discover the wide diversity of biosurfactants The aim of this review is to report fundamentals for methods and techniques for qualitative and cuantitative analysis of microbial biosurfactants. It is aimed at students, biotechnologists and other experts interested in exploring tensoactive molecules of glycolipidic nature. It also contains detailed information about the solids culture media used (specific and selective), different methods to recover these expolymers: acid precipitation and organic solvent extraction. Finally in this review it includes the most innovative and comprehensive methods used for chemical characterization of biosurfactants: spectroscopy UV/Vis, infrared spectroscopy, nuclear magnetic resonance, gaseous and liquid chromatography and mass spectrometry.

Keywords: Rhamnolipids, surface tension, emulsifiers, biotechnological applications

INTRODUCTION

The biosurfactants (BS) are extracellular compounds produced mainly by fungi and bacteria **(Banat *et al.*, 2000)**. These molecules have emulsificant and dispersant properties, they achieve reduce water surface tension from 72 to 25 mN/m approximately **(Supaphol *et al.*, 2011)**. The microbial biosurfactants can enhance bioavailability and biodegradability of low solubility compounds **(Chrzanowski *et al.*, 2012)**, for this reason they have been applied in bioremediation processes of polluted environmental sites with heavy metals, organophosphate pesticides and hydrocarbon total petroleum **(Banat *et al.*, 2000; Ortíz-Hernández *et al.*, 2001; Yañez-Ocampo *et al.*, 2009; Yañez Ocampo *et al.*, 2011)**. In addition, BS have applications in the alimentary, pharmaceutical and cosmetical industry **(Singh *et al.*, 2007; Sajna *et al.*, 2013)**.

For the biotechnological production of BS there are two stages to consider, first microbial strains potentially producers of BS must be explored, isolated and adapted by selective and differential media **(Konishi *et al.*, 2015)**, in this stage it is important to carry out several assays in order to know the emulsion index as well as tensoactive, hemolytic and dispersant activity **(Cassidy and Hudak, 2001; Kitamoto *et al.*, 2002)**. In addition, it is required the research of the optimal culture conditions in liquid medium such as pH, agitation speed, temperature and selection of the carbon source for the culture medium. In this sense nowadays there is an interest for using low cost carbon sources such as soja oil, corn oil used, glycerol, lacto serum, coffee waste, and others hydrophobic compounds by example n-decane, n-tetradecane, paraffin **(Abbasi *et al.*, 2012; Abbasi *et al.*, 2013)**. The second stage refers for their production and industrialization; the costs can be for approximately 60% of the total production cost, because of the unitary operations to obtain BS **(Makkar *et al.*, 2011; Reis *et al.*, 2013)**. The BS are extracellular products, therefore techniques used to separate and recover, depend of its biochemical nature and the selection of the kind method to isolated them **(Sen and Swaminathan, 2005)**. The most common techniques are acid precipitation and extraction with organic solvents.

After extraction it is necessary to purify and characterize the tensoactive, this is achieved by applying different methods; chromatographic and spectroscopic **(Smyth *et al.*, 2010; Makkar *et al.*, 2011; Thavasi *et al.*, 2011; Kuyukina *et al.*, 2013)**. The optimal use of new analytic instruments, requires basic comprehension and knowledge of principles for these specific measurement systems, in order to obtain a highly tensoactive molecule.

About this last topic the molecular characterization of BS, it is known that they are amphiphilic molecules of low molecular weight, mainly glycolipids and lipopeptids, the polar region or hydrophilic is a carbohydrate (rhamnose, trehalose, mannose) and the non polar region or hydrophobic is a hydrocarbonated chain of long variable (fat acids saturated and unsaturated). Figure 1 show the principal chemical structures of BS best studied **(Banat *et al.*, 2000; Banat *et al.*, 2010; Abdel-Mawgoud *et al.*, 2010; Müller *et al.*, 2012)**.

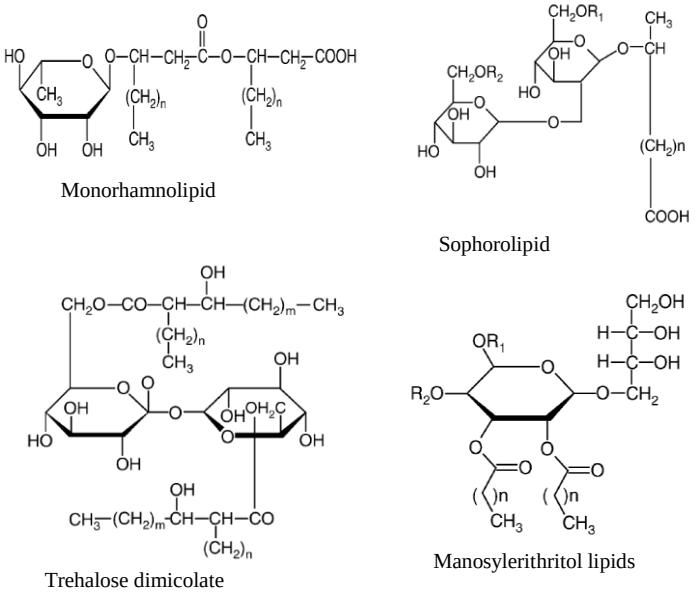

Figure 1 Chemical structures of the best studied microbial biosurfactants **(Banat *et al.*, 2010)**

Several species of *Rhodococcus* sp. can produce trehalolipids, also some yeast as *Candida* sp. can synthesize manosylerithritol lipids, *Bacillus subtilis* produce a BS known as surfactin that currently is used for industrial purposes with pharmaceutical and alimentary applications **(Al-Bahry *et al*., 2013)**. The aim of this paper is to contribute with a review about the recent methods to select and adapt strains producers of biosurfactants, as well as its analytical spectroscopic methods and chromatographic to elucidate quantitative and qualitatively its structure.

FUNDAMENTALS FOR ISOLATION OF MICROORGANISMS PRODUCERS OF BIOSURFACTANTS

In several environments like sea, mangrove sediments or soil polluted with oil hydrocarbon or pesticides, the microorganisms produce biosurfactants, mainly when they are exposed to low solubility carbon sources **(Souza *et al*., 2014)**. In vitro conditions, is known that an excess in carbon source (by example glucose, glicerol or frying oils used) and stress with low levels of nitrogen, promotes rhamnolipids production **(Lee *et al*., 2008; Arutchelvi *et al*., 2011; Kryachko *et al*., 2013)**. The group of *Pseudomonas* sp., are producers of mono and dirhamnolipids, their biosynthesis pathway involves rhamnosyltransferases RhlB y RhlC mainly. Several reports indicate that rhamnolipids production in *P. aeruginosa* is strongly controlled by genetic regulation (transcriptional and postranscriptional) of the rmlBDAC and rhlAB operons called quorum sensing, it is a bacterial communication system characterized by the secretion and detection of signal molecules – autoinducers – within a bacterial population **(Soberón-Chávez *et al*., 2005; Müller *et al*., 2012; Reis *et al*., 2013; Cortés-Sánchez *et al*., 2013)**.

QUALITATIVE METHODS FOR BACTERIAL BIOSURFACTANTS DETECTION

The most reported assays for detection of bacteria producers of biosurfactants, mainly of glycolipidic nature are described briefly in this section.

Agar hexadecyltrimethylammonium bromide (CTAB) assay

This method can detect the production of anionic biosurfactant, rhamnolipids specifically. The medium used for this purpose is an agar based on mineral salts, a low solubility carbon source (v. gr. glycerol, vegetable oil, hydrocarbon), blue methylene and CTAB. The CTAB is a cationic salt that reacts with the rhamnolipid, this complex (CTAB-rhamnolipid-blue methylene) can be visualized by presence of traslucid halo around the colony growth on the agar plates (Figure 2) **(Chandankere *et al*., 2013)**. CTAB can be replaced with N-cetylpyridinium chloride, benzethonium chloride or alkylbenzyldimethylammonium chloride, $CaCl_2$ or $AI(OH)_3$ also can be used instead of CTAB. The blue methylene can be substituted for several dyes like fuchsin, safranin or dichlorphenolindophenol **(Youssef *et al*., 2004)**.

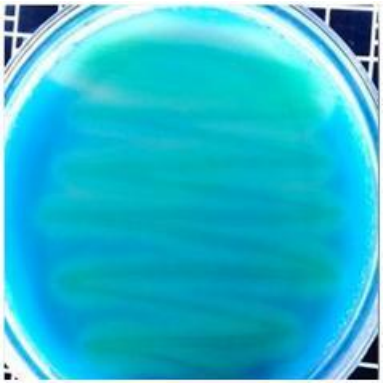

Figure 2 Halo around the bacterial growth agar with methylene blue

Hemolytic activity assay

This method can visualize indirectly the biosurfactants production by inoculating of bacterial strain on a blood agar plate (5 % v/v), incubate to 30 ºC for 24-48 h. The presence of traslucent halos around to growth on the agar plates indicates BS presence with hemolytic activity **(Hassanshahian, 2014)**. However, some bacteria release proteases, or beta-hemolysins, so this test often leads to a positive false result. Besides, not all biosurfactants have hemolytic activity, for this reason it is necessary to perform more tests to confirm BS production **(Youssef *et al*., 2004; Zhang *et al*., 2012)**.

Drop collapse test

This method is quick and easy to detect of BS presence in a sample of bacterial liquid medium culture. By applying a drop of cell free supernatant (BS crude), on a set of hydrophobic phases (v. gr. mineral oil, soja oil, motor oil) it is possible to see the collapse of the oil drop (Figure 3). Additionally it is necessary to run several positive controls, using synthetic surfactants as SDS or tween 80 and try different concentrations. For more details about this assay it is recommended to consult to **Tugrul and Cansuna, (2005); Abdel-Mawgoud *et al*., (2011)**.

Figure 3 *Pseudomonas aeruginosa* supernatant on the oily surface it is collapsed **(Tugrul and Cansunar, 2005)**

Oil displacement test

This technique consist in adding 50 mL of destilled water in a Petri dish, 100 µL of oil and from 5 to 30 µL of cell free supernatant **(Affandi *et al*., 2014; Hassanshahian, 2014)**. The tensoactive activity can be measured with the diameter of the oil displaced after addition of BS. The increased of the diameter is directly related to a high tensoactive activity. This test provides indirect information about biological compounds with tensoactive activity. Figure 4 shows a representative study.

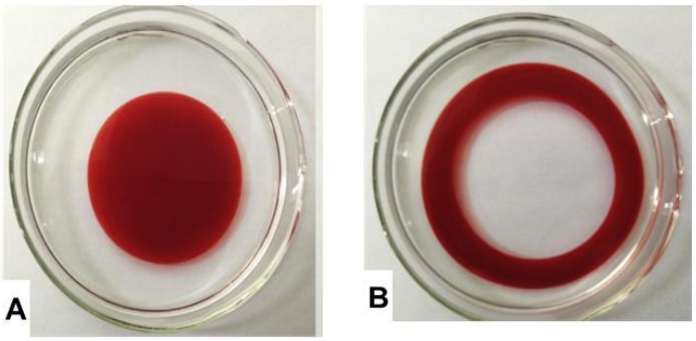

Figure 4 Oil drop displacement A) red oil drop before the experiment B) after applying cell free supernatant (5µL) **(Liu *et al*.,2015)**

Emulsion index (IE_{24})

The emulsification index consist in adding in an screw-cap test tube a mixture (1:1 v/v) of a hydrocarbon (motor oil, corn oil, cotton oil, n-hexadecane, diesel or kerosene) **(Dubey *et al*., 2012)** and cell-free culture broth, after a vigorous homogenizing for two minutes in a vortex, the mixture is left to repose for 24 h to see a stable emulsion layer (Figure 5) **(Ayed *et al*., 2015)**. The emulsification index is calculated by dividing the height of the emulsion layer by the total height of the mixture and multiplying by 100 **(Hassanshahian, 2014; Luna *et al*., 2015)**. In this test, it is also necessary to perform the same positive controls cited above.

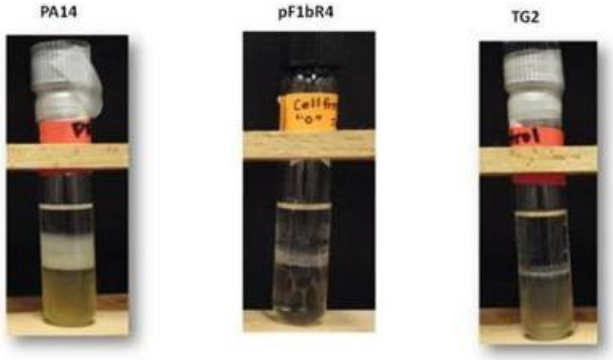

Figure 5 Emulsification of hexadecane by *P. aeruginosa* PA14, *E. coli* pF1bR4 and *E. coli* TG2 **(Arutchelvi *et al*., 2011)**

Determination of Surface tension

This parameter is measured using a digital tensiometer with a Du Noüy ring or Wilhelmy plate. Both methods quantify the strenght required to separate the ring or platinum plate from the liquid surface. The strength is reported in mN/m, this indicates the surface tension the liquid containing BS. Information detailed about specific procedures are available in **Abdel-Mawgoud *et al*. (2011); Burgos-Díaz *et al*. (2013); Xiao *et al*. (2013); Ayed *et al*. (2015)**. A typical parameter for studying biosurfactants is the Critical Micellar Concentration or CMC, who means the concentration of biosurfactant required for micella formation and also reach the lower value of surface tension. Table 1 shows CMC values for various biosurfactants, organized by ascending order, from lowest to highest value. The ideal biosurfactant must have a low value of CMC and also achieve a minimum surface tension **(Mao *et al*., 2015)**.

Table 1 Values of CMC and surface tension published for biosurfactants produced by microorganisms

Microorganism	Biosurfactant	CMC (g/L)	Surface tension (mN/m)	Reference
Bacillus subtilis	Surfactin	0.017	27.2	**Sen and Swaminathan, 2005**
Bacillus subtilis PT2	Surfactin	0.025	26.4	**Pornsunthorntawee *et al*., 2008**
Pseudomonas fluorescens BD5	Lipopeptid	0.072	31.5	**Janek *et al*., 2010**
Pseudomonas aeruginosa SP4	Monorhamnolipid	0.120	28.3	**Pornsunthorntawee *et al*., 2008**
Rhodococcus spp. MTCC 2574	N/I	0.120	30.8	**Mutalik *et al*., 2008**
Sphingobacterium sp.	Phospholipid/ Lipopeptide	6.3	22	**Burgos-Díaz *et al*., 2011**
Streptococcus thermophilus	Glycolipid	20	36	**Rodrigues *et al*., 2006**

N/I= not identified, CMC= Critical Micellar Concentration

EXTRACTION METHODS OF BIOTENSOACTIVE COMPOUNDS

After incubation time in a medium of biosurfactants production, it is necessary to select a method for their extraction. In this section, the most common methods used for this purpose are reported.

Extraction by centrifugation

Through centrifuge force, is possible to separate biomass from the culture medium to obtain a cell-free supernatant, which contains total biosurfactants. Table 2, shows different speeds centrifugation to separate the biomass from the culture broth and obtain the supernatant with total biosurfactantes.

Table 2 Speed centrifugation to extract total biosurfactants from biomass in the culture medium

Speed *g*	Centrifugation time (min)	Temperature (ºC)	Type of biosurfactant	Reference
5000	20	4	Lipopeptide	**Rufino *et al*., 2014**
7,656	10	N/A	Trehalolipid	**Bajaj *et al*., 2014**
8,000	15	4	Lipopeptide and Glycolipids	**Burgos-Díaz *et al*., 2011**
8,643	20	N/A	Lipopeptide	**Sarafin *et al*., 2014**
9,690	30	4	Rhamnolipids	**Aparna *et al*., 2012**
10,000	15	4	Rhamnolipids	**Abbasi *et al*., 2012**
10,800	20	4	Ochrosin	**Kumar *et al*., 2014**
10,956	10	N/A	Surfactin	**Liu *et al*., 2015**
11,952	20	4	Lipopeptide	**Xia et al., 2014**
11,952	10	4	Rhamnolipids	**Bharali *et al*., 2013**
11,952	15	4	Rhamnolipids	**Zou *et al*., 2014**
14,000	5	N/A	Rhamnolipids	**Costa *et al*., 2011**
20,217	20	4	Glycolipopeptid	**Jain *et al*., 2013**

N/A= data not available

Extraction by acid precipitation

Once obtained the cell-free supernatant, it is necessary to recover the mixture of biosurfactants, for this purpose a method commonly used is the acid precipitation. The supernatant must be acidified with 2 N HCl until pH 2.0 and is kept overnight at 4 ºC. The precipitate obtained is recovered by centrifugation and the pellet is washed with acidic water (pH 2.0 with HCl) and then washed with alkaline water (pH 11.0 with NaOH) to achieve a final pH 7.0. The precipitate is dried with heat **(Salleh *et al.*, 2011)**. Table 3 shows several reports about the extraction of biosurfactants with a mixture of both methods: acid precipitation and extraction with solvent.

Extraction by organic solvent

This technique is used in combination with acid precipitation to obtain higher yields of extraction. The supernatant is acidified in separatory funnel with HCl to pH = 2.0, after equal volume of solvent is added, both liquids are agitated, allowing to stand until observe two separate phases, the organic phase is recovered and placed it in a rotary evaporator until observe an extract of brown colour. The organic solvent extraction after acid precipitation is the most applied method to recover approximately 90 % of BS **(Gusmão *et al*., 2010; Salleh *et al*., 2011)**. The solvents used in the extraction can be recovered and reused, this represents good laboratory practice.

Rufino et al. **(Rufino *et al*., 2014)** reported BS recovering from the culture of *Candida lipolytica* UCP 0988, using the mixture chloroform/culture broth (1:1 v/v), with a yield of 8.0 g/L of tensoactive. It has also reported BS extraction with a mixture ethyl acetate/ methanol (8:1 v/v) and chloroform/methanol (2:1) v/v recovering 10 g/L BS crude **(Burgos-Díaz *et al*., 2011; Affandi *et al*., 2014)**.

Extraction by Lyophilization

Lyophilization is also a methodology used to recover BS. In this technique, the first step is to obtain a cell-free supernatant by centrifugation, after the exopolymer is precipitated by adjusting pH = 2.0 with chlorhydric acid overnight at 4 ºC. The pellet precipitated is recovered by centrifugation and it is resuspended in distilled alkaline water (pH = 8.0) and then is lyophilized to obtain a brown powder. Through this method, **Al-Bahry *et al*. (2013)** recovered 2.29 g/L of BS from a culture of *Bacillus subtilis* B20, also **Xiao *et al*. (2013)** produced BS with *Klebsiella* sp., in their research, were recovered 10.1, 5.1, 3.25, 3.1, 2.75 and 2.62 g/L of BS produced with starch, sucrose, xylose, galactose, glucose and fructose respectively.

SPECTROPHOTOMETRIC METHODS TO QUANTIFY BIOSURFACTANTS

Spectrophotometric methods are used to determine the presence of biosurfactants in either the culture medium or with the compounds extracted. The presence of sugars in the BS can be identified by different methods spectrophotometrics such as anthrone, phenol-sulfuric, orcinol and DNS **(Smyth *et al*., 2010; Abdel-Mawgoud *et al*., 2011)**. However, interferences from chemicals and carbon sources can result in inaccurate results and therefore should only be used as a rough indicator of biosurfactant production.

Anthrone Assay

This assay can be used for all types of glycolipid biosurfactants, it detects the amount of carbohydrate present. With the anthrone reagent previously prepared, add 200 µL of cell-free supernatant and 1000 µL of anthrone, heat the sample for 9 min, then measure by absorbance at 625 nm **(Smyth *et al*., 2010)**. The anthrone assay can be used to detect of glycolipid in the culture broth.

Phenol-sulphuric acid assay

The phenol-sulphuric acid reagent is widely used as a chemical method for the measurement of the sugars of polysaccharides, glycoproteins and glycolipids. The method is sensitive for determining small quantity of sugars in biological samples. Add 2 mL of supernatant in the test tube, add 1 mL of phenol (5%) and 5 mL of sulfuric acid concentrated, measure after 30 min at 490 nm **(Rufino *et al*., 2014)**.

Orcinol assay

This colorimetric assay is based on the reaction of orcinol (1,3-dihydroxy-5-methylbenzene) and the sugar moiety under acidic conditions and high temperature to produce a blue-green colored dye whose absorbance can be measured at 665 nm and used to roughly quantify the glycolipid concentration in the sample **(Smyth *et al*., 2010; Abdel-Mawgoud *et al*., 2011)**.

DNS assay

Is based on a redox reaction between 3,5 dinitrosalicilic acid and reducers sugars in supernatant free cell culture. In order to determine sugar concentration, 3 mL sample with 5 mL of chlorhydric acid (HCl 6 N) are mixed in glass tubes and heated to 65 ºC by 10 min after it is cooled and neutralized with sodium hidroxide (NaOH), subsequently reactant DNS is added. Absorbance is measured in a spectrophotometer at 540 nm; is necessary to build a standard curve, the result is expressed in g/L **(Smyth *et al*., 2010; Abdel-Mawgoud *et al*., 2011)**.

Table 3 Extraction methods for recover biosurfactants by using acid precipitation and organic solvents

Acid precipitation conditions	Solvent used	Microorganism producer of biosurfactant	Yield (g/L)	Reference
H_2SO_4 1 M pH 2.0	Chloroform/ Methanol (2:1v/v)	*Pseudomonas* sp. 2B	4.97	**Aparna *et al*., 2012**
HCl 6 M pH 2.0 Overnight at 4 ºC	Methanol	*Bacillus subtilis* BS-37	0.585	**Liu *et al*., 2015**
HCl 2 N pH 2.0	Ethyl acetate	*Ochrobactrum* sp. BS-206 (MTCC 5720)	0.28	**Kumar *et al*., 2014**
HCl 2 N pH 2.0 Overnight at 4 ºC	Ethyl acetate	*Rhodococcus* sp. IITR03	N/A	**Bajaj *et al*. 2014**
HCl 6 M pH 2.0 Overnight at 4 ºC	Methanol	*Candida sphaerica* UCP0995	9	**Luna *et al*., 2015**
HCl 6 N pH 2.0	Chloroform/ Methanol (65:15 v/v)	*Kocuria marina* BS-15	0.00197	**Sarafin *et al*., 2014**
HCl 6 N pH 2.0 Overnight at 4 ºC	Ethyl acetate	*Pseudomonas aeruginosa* MA01	12	**Abbasi *et al*., 2012**
HCl 6 N pH 2.0 Overnight at 4 ºC	Ethyl acetate	*Pseudomonas aeruginosa* OBP1	N/A	**Bharali *et al*., 2013**
HCl 6 N pH 2.0 Overnight at 4 ºC	Chloroform/ methanol (2:1 v/v)	*Pseudomonas* sp.	5.2	**Silva *et al*., 2014**
N/A	Chloroform	*Candida lipolytica* UCP 0988	8	**Rufino *et al*., 2014**
N/A	Ethyl acetate/ methanol (8:1 v/v)	*Sphingobacterium detergens*	0.466	**Burgos-Díaz *et al*., 2013**

N/A= data not available

METHODS AND TECHNIQUES FOR BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF BIOSURFACTANTS

The analytical chemistry employs several quantitative and qualitative methods to elucidate the chemical structure of organic compounds. The qualitative method reports the identity of the atomic species and functional groups of the sample. The quantitative method gives the numeric information about the relative quantity in one or several components.

The main methods to study the biochemical composition and molecular characterization of BS are described next, such as Fourier transform infrared spectroscopy (FTIR), nuclear magnetic resonance (NMR), also chromatographic methods like thin layer chromatography (TLC), high performance liquid chromatography (HPLC) **(Swaathy *et al*., 2014)** coupled with a light scattering detector (ELSD), mass spectrometry coupled with high performance liquid chromatography (HPLC–MS) or gas chromatography (GC–MS) **(Ribeiro *et al*., 2012)**. Table 4 reports the methods used to characterize BS, mainly rhamnolipids.

Table 4 Methods for the qualitative and quantitative study of biosurfactants

Instrumental method	Type of biosurfactant	Reference
Infrared Spectroscopy (IR)	Glycolipids, rhamnolipids, xylolipid, lipopeptids	**Jain *et al*., 2013; Vecino *et al*., 2013; Chandankere *et al*., 2013; Jain *et al*., 2012; Sharma *et al*., 2015; Nalini and Parthasarathi, 2014; Jara *et al*., 2013; Aparna *et al*., 2012; Pantazaki *et al*., 2011; Ibrahim *et al*., 2013; Ismail *et al*., 2013**
Nuclear Magnetic Resonance (NMR)	Sophorolipid, xylolipid	**Konishi *et al*., 2015; Sharma *et al*., 2015**
Thin layer chromatography (TLC)	Lipopeptids, glycolipids	**Sharma *et al*., 2015; Burgos-Díaz *et al*., 2011; Xia *et al*., 2014; Pedetta *et al*., 2013**
High Performance Liquid Chromatography (HPLC)	Rhamnolipids, xylolipid	**Sharma *et al*., 2015; Pantazaki *et al*., 2011; Compaoré *et al*., 2013**
Column Chromatography (CC)	Lipopeptids ,glycolipids	**Burgos-Díaz *et al*., 2011**
Gas chromatography (GC)	Xylolipid	**Sharma *et al*., 2015**
Mass spectrometry (MS)	Rhamnolipids, xylolipid	**Gudiña *et al*., 2015; Sharma *et al*., 2015; Pantazaki *et al*., 2011**

Infrared spectroscopy

Nowadays the infrared spectroscopy is used as a technique to identify organic molecules like biosurfactants, through the vibrations and frequences characteristics of their chemical bonds, in the interval of infrared spectrum. The infrared spectroscopy is based on the infrared radiation absorption by molecules in vibration. Every type of chemical bond absorbs the infrared light at a specific frequence, so it is possible to know what kind of functional groups have the molecules in study. For example, the C-H bonds are localized in the spectrum of 4000-2500 cm^{-1} of wavelength **(Chadwick *et al*., 2014)**.
Each molecule has a typical infrared spectrum like a finger print, so analyzing several wavelengths that a substance absorbs in infrared zone, it is possible to know the functional groups that the glycolipid has **(Madsen *et al.*, 2011; Li *et al*., 2014)**. It is based on the relatively broad IR absorption bands corresponding to various hydroxyl, ester, and carboxylic groups present in glycolipids. This method has been used for the quantification of complex RL mixtures, but it suffers from interferences by other constituents in the medium and of changes in pH **(Rikalović *et al*., 2012)**.

Nuclear Magnetic Resonance

The previously reported methodologies allow the identification of molecular structure to quite a high extent though not completely. To achieve a full structural determination, NMR needs to be utilized and it is the most powerful method, this is able to identify functional groups as well as the position of linkages within the carbohydrate and lipid molecules. Using a series of NMR experiments the exact location of each functional group can be obtained and information about the structural isomers is also possible **(Smyth *et al*., 2010)**.
Nuclear magnetic resonance (NMR) spectroscopy is a powerful tool for structural elucidation, which is obtained in 1D and 2D experiments. In organic chemistry, the main tools for structural elucidation are 1H and 13C NMR spectra associated with 2D experiments, such as 1H–1H COSY, 1H–1H TOCSY, 1H–13C HMQC, 1H–13C HMBC and NOESY. The glycolipids should be dissolved in dueterated chloroform and a series of 1D (1H and 13C) and 2D (such as COSY, ROSY, HMQC and HMBC) experiments should be carried out by NMR. Specific details with regard to the results for rhamnolipids, sophorolipids, trehalose lipids and mannosylerythritol lipids can be obtained from Smyth et al. **(Smyth *et al*., 2010)**.

Chromatography

This method is used to separate organic compounds from complex mixtures. Chromatography is based in a mobile phase which transport the sample, the mobile phase can be gas or liquid, then sample is transported into a stationary phase is a column, every compound of the mixture in the sample is distributed. The compounds of the sample are separated, by this process the biosurfactants can be analized qualitatively or quantitatively **(Sen and Swaminathan, 2005; Abbasi *et al*., 2013)**.

Thin layer chromatography

Thin layer chromatography (TLC) is a simple method allowing detection of glycolipids and can also provide information on possible structural types of glycolipids present. TLC detection should be carried out before purification procedures to determine the presence of glycolipids and can also be used to determine purity after purification steps **(Smyth *et al*., 2010; Abdel-Mawgoud *et al*., 2011)**.
TLC can be used as a preliminary method for evaluation and/or screening of SLs production. A stationary phase of silica GEL 60 or silica GEL F254 is frequently used in association with chloroform/ methanol/water mobile phase. For detection of glycolipids bands by TLC, several staining solutions in association with heat have been used and examples are anthrone-naphtol and p-anisaldehyde **(Ribeiro *et al*., 2012)**.

Gas chromatography

Specifically rhamnolipids cannot be directly analyzed by gas chromatography (GC) because of their relatively high molecular weight. Prior to analysis, rhamnolipids are hydrolyzed with acid, their acid groups are modified into methyl esters, then rhamnose and various 3-hydroxyfatty acids can be analyzed, identified and quantified using flame ionization detection by GC **(Abdel-Mawgoud *et al*., 2011)**.

High Performance Liquid Chromatography (HPLC)

HPLC is a method that allows the separation of glycolipids **(Compaoré *et al*., 2013)**. This method is especially well-suited for analysis of rhamnolipids, it is generally performed using C8 or C18 reverse-phase columns with a water/acetonitrile gradient **(Hassanshahian, 2014)**. HPLC-UV can also be used for analysis when the test compounds have been derivatised to p-bromophenacyl esters **(Smyth *et al*., 2010)**. HPLC-UV and HPLC-ELSD both require comparison with retention times of standards to allow identification of the structure, however, the presence of isomers cannot be detected by Gas chromatography.

Mass spectrometry

The mass spectrometry analysis can be used to identify the chemical structures of biosurfactant mixtures **(Rikalović *et al*., 2012)**. Electrospray Ionization (ESI) is used to ionize BS prior to mass analysis. The structures of the mono- and di-rhamnolipids were confirmed by electrospray tandem mass spectrometry (ESI–MS/MS). The molecular weight of the pseudomolecular ion [M-H] can be directly obtained. This provides some information on the nature of the BS congener eluting from the column at that retention time **(Abdel-Mawgoud *et al*., 2011)**. Several strains of *P. aeruginosa*, produce mixtures of rhamnolipids of different congeners, being the most common L-rhamnosyl-bhydroxydecanoyl- b-hydroxydecanoate (Rha-C10-C10) and Lrhamnosyl- L-rhamnosyl-b-hydroxydecanoyl-b-hydroxydecanoate (Rha-Rha-C10-C10) **(Aparna *et al*., 2012)**. Other congeners frequently found include mono- and di-rhamnolipids with acyl chains containing 8, 10, 12 or 14 carbons, mostly saturated, and, less often, containing one or two double bonds, as well as with only one b-hydroxy fatty acid [42, **(Gusmão *et al*., 2010; Ibrahim *et al*., 2013; Janek *et al*., 2013; Hoškova *et al*., 2013)**.

Table 5 Chemical composition of rhamnolipid mixture produced by *Pseudomonas* sp. 2B culture determined by MS analysis **(Aparna *et al*., 2012)**

Rhamnolipid congeners	Pseudomolecular ion (*m/z*)
Rha-Rha- C_8-C_{10}	621.0
Rha-Rha- C_{10}-C_{10}	650.0
Rha-Rha- C_{10}-C_{12}	678.0
Rha-C_{10}-C_{10}	505
Rha- C_{10}-C_{12}	532
Rha-C_{10}	333
Rha- C_{10}-C_{10}	504
Rha-Rha- C_{12}-C_{10}	678
Rha-Rha- C_{10}	479

CONCLUSION

The biotechnological production of biosurfactants can be feasible with use of carbon sources low cost like organic wastes from agriculture or vegetable used oils. The biosurfactants production to an industrial scale remains challenge, to substitute the synthetic surfactants. Despite their environmental advantages and equal performance, commercialization of these molecules remains a challenge. The latter issue can partially be tackled by screening for the research of better producers and optimizing the fermentation process. In order to reduce cost of production of biosurfactants, there are several methods to extract them from culture medium, like centrifugation or acid precipitation. For environmental applications of biosurfactants, it requires only biosurfactant crude from a free cell culture. For scientific and patent creation purposes it is necessary to have a complete characterization, therefore, spectroscopic, chromatographic and mass spectrometry methods are accurate powerful tools to elucidate novels biosurfactants.

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