Essay

Lipopeptides produced by biological control agents of the genus *Bacillus*: a review of analytical tools used for their study

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Abstract

The genus *Bacillus* has a wide metabolic diversity associated with its prevalence in various ecosystems. At present, one of the most studied characteristics of this bacterial genus is its ability to reduce the incidence of diseases in agricultural crops, due to the direct or indirect repression of the growth of the causative agents of said diseases. Among the most studied mechanisms in the biological control of strains of the genus *Bacillus* against phyto-pathogenic agents, lipopeptides, such as surfactins, iturins and phengicins, which are low molecular weight compounds with amphiphilic characteristics that provide protection to plants both in preconditions as post-harvest. Lipopeptides can directly antagonize phytopathogenic agents and induce systemic resistance strategies in associated plants. However, the wide use of lipopeptides in the agricultural, pharmaceutical, or food sector depends on the development of efficient and economical analytical processes for the extraction, identification, and quantification of said bioactive compounds. This test presents an analysis of various analytical procedures used for the extraction, and qualitative and quantitative identification of these metabolites.

Keywords: Bacilli, biocontrol, lipopeptides.

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By 2050 it is predicted that the agricultural sector will need to increase its productivity between 70 and 100%, in order to meet the food demand of the human population, which will amount to ~10 billion people (FAO, 2017). However, one of the main limitations for the increase in agricultural production is the incidence of crop diseases caused by phyto-pathogens, causing an average yield decrease of 15-25% (Villa-Rodríguez *et al*., 2016; Gupta *et al*., 2018; Parra-Cota *et al*., 2018; Díaz-Rodríguez *et al*., 2019).

Therefore, the use of pesticides worldwide has increased from ~1.3 kg ha^{-1} to 2.57 kg ha^{-1} from 1992 to 2016; that is, 197.69% (García-Gutiérrez *et al*., 2012; FAO, 2018); however, only 0.1% reaching the desired objective (agricultural cultivation), where the remaining volume is mobilized by contaminating the surrounding environment, affecting other organisms, being chemically complex substances, severely impacting the environment generating pollution of soils and aquifers, soil degradation and salinization, loss of biodiversity and toxicity effects of remaining components, chemical or microbial degradation, in addition to generating loss of biodiversity (Gálvez-Gamboa *et al*., 2018).

Therefore, the generation of agro-biotechnological alternatives for the control of phyto-pathogens is decisive to meet the demand for current and future food in a sustainable way, focused on preserving the fertility of agricultural soils, and reducing potential effects on the environment (de los Santos Villalobos *et al*., 2018; Robles-Montoya *et al*., 2020).

Globally, the interest of the agricultural sector has increased in the use of phyto-pathogen control strategies by Biological Control Agents (ACB), particularly bacteria, as observed by the exponential increase in the world market for biopesticides, of $ 800 million in 2014 to $ 2.8 billion today (Villa-Rodriguez *et al*., 2019), with an estimated increase of 15% to 20% by 2020 (Gomez *et al*., 2018). ACB are beneficial organisms, their genes and/or products, such as metabolites, to reduce the negative effects of plant pathogens through antagonistic actions by different mechanisms, including mycoparasitism, lytic enzyme production, induction of systemic response of the plant and production of δ-endotoxins, siderophores and lipopeptides (Robles-Montoya *et al*., 2019; de los Santos Villalobos *et al*., 2019; Valenzuela-Ruiz *et al*., 2019; Villa-Rodríguez *et al*., 2019).

This led to the study of various microbial strains such as ACB, among which the genus *Bacillus* has been widely studied for its ability to sporulate, which facilitates its production and storage as a bioinoculant for long periods of time, in addition to its abundance, diversity and ubiquity in various agro-systems (soil, water and plant), its population being significantly larger compared to other microbial genera (Villarreal-Delgado *et al*., 2018; de los Santos Villalobos *et al*., 2019; Santoyo *et al*., 2019).

Additionally, the Bacilli unleash due to their diverse metabolic capacities, producing antibiotic antimicrobial metabolites, lytic enzymes, chitinases, cellulases, glucanases, and lipopeptides (Valenzuela-Aragon *et al*., 2019). Lipopeptides are low molecular weight compounds with amphiphilic characteristics that provide protection to plants both in pre-harvest and post-harvest conditions, by directly suppressing the growth of pathogens or inducing systemic resistance in host plants (Hashem *et al*., 2019).

Recently, Coutte *et al*. (2017) found 263 different lipopeptides synthesized by 11 microbial genera. Within these, *Bacillus* represents the most abundant producer with 98 different lipopeptides (classified in families), where in the context of biocontrol of plant diseases, the three families of *Bacillus* lipopeptides: surfactins, iturins and fengycina are of interest for their activity antagonist for a wide range of potential phytopathogens, including bacteria, fungi and oomycetes (Ongena and Jacques, 2008). The objective of this trial is to describe critically various analytical procedures used for the study (including the extraction and qualitative and quantitative identification) of lipopeptides produced by the genus *Bacillus*, focused on the production of efficient and sustainable biopesticides.

Methods used for the extraction, identification and purification of lipopeptides

Extraction and purification of lipopeptides

Extraction and purification are key stages in the study and commercialization of lipopeptides (Coutte *et al*., 2017). Generally, the purification of these compounds starts from a liquid matrix (eg the culture broth where the *Bacillus* strain was grown), although its production in solid state fermentation has also been reported (Su *et al*., 2018).

The purification methods are based on the physicochemical properties of lipopeptides (polarity and molecular weight). Frequently, a single method is not sufficient for the purification of these compounds. For this reason, it is common for lipopeptide purification to include multiple stages, applying methods such as i) acid precipitation; ii) use of organic solvents; iii) ultrafiltration; iv) solid phase extraction; and v) chromatography (Table 1).

Table 1. Methods of extracting and purifying lipopeptides from strains of the genus *Bacillus*.

Species	Method	Description	Reference
B. amyloliquefaciens	Acid precipitation	Acid precipitation with HCl at pH 2 overnight.	Hsieh *et al*. (2004)
B. subtilis	Acid precipitation	HCl precipitation overnight, centrifugation, and drying at 70 °C	Abdel-Mawgoud *et al.* (2008)
B. velezensis	Solvents, liquid-liquid extraction	The metabolites were isolated from a liquid LB culture grown at 28 °C for 72 h by ethyl acetate	Grady *et al.* (2019)
B. subtilis	Solvents, liquid-liquid extraction	The supernatant obtained from a liquid culture was mixed with a volume (1:1) of ethyl acetate.	Alajlani *et al.* (2016)
B. mojavensis	Ultrafiltration	The purified lipopeptides were obtained using a 10-kDa membrane	Hmidet *et al.* (2017)
B. megaterium	Ultrafiltration	Metabolic extracts of lipopeptides were purified using ultrafiltration with a pore size of 30-kDa	Ma *et al.* (2016)
B. subtilis	Solid phase extraction	A C_{18} column was used as a solid phase and a gradient of acetonitrile as a solvent to elute lipopeptide fractions.	Alajlani *et al.* (2016)

Acid precipitation

One of the most common methods to start the lipopeptide purification is by acid precipitation, using concentrated hydrochloric acid (HCl) (Abdel-Mawgoud *et al*., 2008). This method consists in lowering the pH (~2) of the culture broth in which these compounds are dissolved. In this way, the negative charges of the lipopeptides are neutralized, decreasing their solubility in the aqueous phase, resulting in their precipitation (Biniarz *et al*., 2016).

Once lipopeptides precipitate, they can be recovered for further analysis or even to be subjected to other purification steps. Acid precipitation is a very effective and economical method to isolate lipopeptides. Although the use of this method allows recovering ~97% of the lipopeptides present in the culture medium, its main disadvantage is that it is not a selective method, since other compounds (eg. peptides) can coprecipitate with the lipopeptides, obtaining purities below 60% (Coutte *et al*., 2017).

Organic solvents

The use of organic solvents is one of the most used methods for the extraction and purification of lipopeptides. Solvents with different polarity indices such as chloroform, methanol, petroleum ether, ethyl acetate, n-hexane and ether have been used for this purpose (Biniarz *et al*., 2016). The use of these solvents can be used as a complementary method to acid precipitation, for example, the precipitate obtained with this method can be subjected to extraction with one of the mentioned solvents (solid-liquid extraction). However, solvent extraction can also be used as an initial step in the purification process, for example, by a liquid-liquid extraction by mixing the culture medium and the organic solvent (Alajlani *et al*., 2016).

Solid phase chromatography and extraction (SPE)

The extraction of lipopeptides by methods such as acid precipitation and solvent use, despite presenting high recovery efficiency, are poorly selective methods. Chromatography and solid phase extraction (SPE) are methods that allow obtaining a high degree of purity. These methods are generally used in the final stages of lipopeptide purification (Coutte *et al*., 2017).

Both chromatography and solid phase extraction consists of passing the lipopeptide mixture through a column, in this way the compounds of interest will interact with the solid phase of the column (resin), while the contaminating compounds will not (Poole, 2003).

Subsequently, lipopeptides that interacted with the resin can be recovered by elution with solvents (eg methanol, ethyl acetate or acetonitrile) (Poole, 2003). The success in the purification of lipopeptides with these methods depends mainly on the type of resin and the solvent mixture. It has been reported that column C_{18} is very efficient for adsorption of lipopeptides, due to the hydrophobic motive of these molecules (Razafindralambo *et al*., 1993).

However, due to the similarity in its structure and physicochemical properties, the purification of specific lipopeptide families is usually a complicated process. However, this has been achieved with reverse phase liquid chromatography methods. For example, recently Luna-Bulbarela *et al*. (2018) was able to purify different isoforms of iturines -with variants in the length of fatty acid- by reverse phase chromatography, using a C_{18} column as the stationary phase and a gradient of acetonitrile as the mobile phase.

Ultrafiltration

Ultrafiltration is a promising approach to the recovery of lipopeptides; however, equipment costs are high, which limits their routine use (Isa *et al*., 2007). Lipopeptides at concentrations above their critical concentration of micelles can be associated to form supramolecular structures such as micelles, which have nominal diameters up to two or three times larger than that of a free molecule (Coutte *et al*., 2017). Taking this principle into account, ultrafiltration methods focused on purifying lipopeptides in the form of micelles have been established.

The separation of lipopeptides by the filtration membrane depends on their molecular aggregation behavior and their ability to form micelles (Jauregi *et al*., 2013). Likewise, the pH and the concentration of lipopeptides are important factors in the formation of micelles (Sen *et al*., 2005). Once the lipopeptide micelles have been purified by ultrafiltration, it is possible to denature it with the use of organic solvents such as methanol or ethanol.

Ultrafiltration has been used successfully for the purification of different families of lipopeptides. For example, in one study this method was used to purify a mixture of surfactins and iturines using 10-100 kDa membranes (Jauregi *et al*., 2013). In general, with this method it is possible to achieve a purity of lipopeptides of up to 95% (Jauregi *et al*., 2013).

Identification and quantification of lipopeptides

The chromatographic methods, in addition to being used in the purification of lipopeptides, are used in their identification and quantification. Methods that use different chromatography variants have been established for lipopeptide analysis, including: thin layer chromatography (TLC) and high resolution liquid chromatography (HPLC) (Kinsella *et al*., 2009; Jamshidi-Aidji *et al*., 2019).

Due to the versatility of chromatography for lipopeptide analysis - allowing its purification, identification and quantification - this analytical tool has been the most used in lipopeptide processing. However, there are other spectroscopic methods that have been used for the analysis of these compounds, such as i) nuclear magnetic resonance (NMR) (Son *et al*., 2016), infrared transmission with Fourier transform (FT-IR) (Kong, 2007) and mass spectrometry (Ma *et al*., 2016).

Thin layer chromatography (TLC)

Thin layer chromatography is a very versatile chromatographic technique that can be used for different purposes in lipopeptide analysis. This technique consists in placing the sample of interest near one end of an aluminum plate coated with a thin layer of an adsorbent (eg. silica gel, cellulose, aluminum oxide). The sheet is placed in a closed cuvette containing one or more mixed solvents (mobile phase). As the solvent mixture rises by capillarity through the adsorbent, a differential distribution of the compounds present in the sample occurs (Ciura *et al*., 2017).

The samples will migrate differentially across the plate, resulting in specific values of the delay factor (Rf). The success in the separation of the compounds depends on the adsorbent (stationary phase) and the solvent mixture (mobile phase) (Ciura *et al*., 2017). TLC has been used to identify families of lipopeptides (Jamshidi-Aidji *et al*., 2019). However, it also results in a quick and simple method to evaluate the purity of these compounds during purification steps.

The identification of lipopeptides using TLC is performed by comparing the Rf value between a standard of lipopeptides and the test sample. For example, Geissler *et al*. (2017) established a method to simultaneously identify surfactins, iturins and phengicins in extracts from different *Bacillus* strains. In this study, the Rf values were specific for each family of lipopeptides, using chloroform/methanol/water (65:25:4, v/v/v) as the mobile phase and silica gel as the stationary phase.

High performance liquid chromatography (HPLC)

High performance liquid chromatography (HPLC) allows a mixture of compounds to be separated and can be used to identify, quantify and purify individual compounds from a mixture (Malviya *et al*., 2010). This technique consists in passing a mixture of compounds -by solvents (mobile phase) at high pressures- through a column that contains a resin inside (stationary phase). Once the compounds pass through the column, they emit signals generated by detectors (eg ultraviolet detector, photodiode matrix detector, mass detector, fluorescence detector, etc.) (Malviya *et al*., 2010).

HPLC has gained popularity among chromatographic techniques and is currently the main option for lipopeptide studies (Biniarz *et al*., 2016). This method even allows different isoforms to be separated from the same family of lipopeptides (eg. variations in the length of the fatty acid or in some amino acid).

The identification of lipopeptides with HPLC is performed by a comparison of the Rt between a standard of lipopeptides and the test sample, while quantification requires a calibration curve using different concentrations of a standard (de Souza *et al*., 2018). In HPLC, identification and quantification are analyzes that are carried out simultaneously. HPLC methods have been developed that allow quantification of different lipopeptide families (Table 2). For example, Yuan *et al*. (2011) optimized chromatographic conditions to quantify iturin A homologues, using the Eclipse XDB-C_{18} column as a stationary phase and an acetonitrile gradient as a mobile phase.

Mass spectrometry (MS)

Mass spectrometry (MS) is an analytical technique that is used for the identification of analytes using the mass-charge (m/z) ratio of ionized compounds (Glish *et al*., 2003). A mass spectrometer is an instrument that basically consists of two elements; i) the source of ionization (eg. electrospray, plasma, laser); and ii) one or more analyzers (TOF, quadrupole) (Glish *et al*., 2003).

The most commonly used mass spectrometry techniques for lipopeptide analysis is matrix-assisted laser desorption-ionization (MALDI) and electrospray ionization tandem mass spectrometry (ESI-MS/MS) (Table 2).

Table 2. Methods of identification and quantification of lipopeptides of strains of the genus *Bacillus*.

Lipopeptides	Method	Description	Reference
Surfactins, iturins, fengicines	TLC	The TLC CAMAG system and a gradient of chloroform-methanol-water 65:25:4 (V/V/V) were used as the mobile phase.	Jamshidi-Aidji *et al.* (2019)
Surfactins, iturins and phengicins	TLC	Silica gel was used as the stationary phase and a mixture of chloroform-methanol-water 65:25:4 (v/v/v) as the mobile phase. The identification was carried out comparing Rf values with commercial standards.	Geissler *et al.* (2017)
Iturine A	HPLC	A C_{18} column was used as stationary phase and acetic acid (1%)-acetonitrile 60:40 (v/v) as mobile phase. Quantification and identification was carried out using a calibration curve and comparison of Rt with a commercial standard.	Yuan *et al.* (2011)
Surfactins, iturins and phengicins	HPLC	A C_{18} column was used as the stationary phase. Two solvents were used; solvent A (formic acid, 0.1%) and solvent B (acetonitrile) as the mobile phase. The lipopeptide identification was carried out with a mass detector (MS) and its quantification with a calibration curve with commercial standards.	de Souza *et al.* (2018)
Surfactin and iturin A	HPLC	A C_{18} column was used as the stationary phase and a mixture of acetonitrile: water as the mobile phase. The concentrations of the mobile phase varied according to the lipopeptide to be quantified. Quantification and identification was carried out using a calibration curve and comparison of Rt with a commercial standard.	Kinsella *et al.* (2009)
Surfactins, iturins and phengicins	HPLC-ESI-MS/MS	A Zorbax SB-C18 column and solvents A (0.1% formic acid) and B (acetonitrile) were used as the mobile phase. The identification of the compounds was carried out by examining MS^2 spectra.	Ma *et al.* (2016)

Lipopeptides	Method	Description	Reference
Iturines	HPLC-ESI-MS/MS	A C_{18} column and solvents A (0.1% formic acid) and B (methanol with 0.1% formic acid) were used as the mobile phase. Iturine identification was carried out by examining MS2 spectra.	Dunlap *et al.* (2019)
Surfactins, iturins and phengicins	MALDI	Dihydroxybenzoic acid was used as the matrix and a mass detection range of 200 to 2000 Da was used.	Sajitha *et al.* (2016)
Iturines	NMR	Using 1D and 2D-NMR, new variants of iturins were identified.	Son *et al.* (2016)

MALDI is a mass spectrometry method that allows the identification of compounds without structurally affecting them (El-Aneed *et al*., 2009). In MALDI, the sample to be analyzed is co-crystallized with a matrix compound, which is typically an organic acid that has little UV light absorption. The mixture is subsequently exposed to laser radiation, which results in the vaporization of the matrix compound that adheres to the analyte.

The matrix compound plays a key role in strongly absorbing laser energy, indirectly causing the analyte to evaporate (Hillenkamp *et al*., 1991). The matrix compound also serves as a donor and acceptor of protons and forces the analyte to ionize positively or negatively. MALDI is especially useful for knowing the molecular weight of purified lipopeptides, because they do not undergo structural changes during ionization.

However, this technique has also been used to know the lipopeptide profile of *Bacillus* extracts (Sajitha *et al*., 2016). ESI-MS/MS is a very complete mass spectrometry method to identify lipopeptides. When ESI-MS/MS is coupled to HPLC (HPLC-ESI-MS/MS), it allows simultaneous separation, identification and quantification of lipopeptide samples, being the most complete instrument for lipopeptide analysis (Ma *et al*., 2016). The quantification and separation of lipopeptides with this instrument has the same principle as HPLC, since this unit is coupled to a mass spectrometer.

The identification of lipopeptides using HPLC-ESI-MS/MS can be done using lipopeptide standards by a comparison between m/z values. However, the really useful thing about using this system is that it allows the identification of lipopeptides without the use of standards (Dunlap *et al*., 2019). This is achieved because with the HPLC-ESI-MS/MS system it is possible to simultaneously obtain the primary mass (MS^1) of a compound - usually the intact compound in the form of an ion - and its secondary mass (MS^2), generated from the compound fragmentation.

Fragmentation of the compound results in a specific mass spectrum for that molecule, which can be compared to a database such as Pubchem and Metlin, to make the compound annotation (dereplication). For example, with HPLC-ESI-MS/MS, it was possible to identify more than 10 homologs of fengicins and surfactins, without the use of commercial standards (Rangarajan *et al*., 2014).

Nuclear magnetic resonance imaging (NMR)

Nuclear magnetic resonance imaging (NMR) is a technique that can provide structural information of molecules in solution with a high resolution (De Faria *et al*., 2011). The determination of structures by NMR can be divided into the following steps: i) establish suitable conditions for recording spectra; ii) measuring a series of 1D (1H and 13C) or 2D (eg. COZY, TOCSY and ROSY) NMR spectra; iii) integrate cross peaks and transformation into upper distance limits (calibration); and iv) evaluation of the quality of the molecular structure (Biniarz *et al*., 2016). NMR is a method used to identify lipopeptides.

This is a tool that allows even determining structural variants in lipopeptides, such as amino acid changes in the peptide motif. For example, Volpon *et al*. (2000) determined variations in the lipopeptide piplastatin using NMR, named accordingly as piplastatin A and piplastatin B. With this tool they identified that these lipopeptides differ by one amino acid at position 6 of the peptide motif.

Infrared transmission with Fourier transform (FT-IR)

Fourier transform infrared transmission spectroscopy (FT-IR) is a fast and economical method to characterize the chemical structure of lipopeptides and identify the functional groups present in these compounds (Walker *et al*., 2009). FT-IR is a physicochemical method based on the measurement of the vibrations of a molecule excited by infrared radiation (IR) at a specific wavelength. Despite some differences in FT-IR spectra between lipopeptides and experimental conditions, the FT-IR instrument is commonly used in a range between approximately 4 000 and 400 cm (Kong, 2007).

This method is used to identify lipopeptides at the family level. For example, this technique was used successfully to identify surfactins in *Bacillus circulans*. The latter was achieved by comparing FT-IR spectra between a surfactin standard and a mixture of purified lipopeptides from *B. circulans* (Son *et al*., 2010).

Conclusions

Currently, lipopeptides produced by strains of the genus *Bacillus* represent a promising and sustainable strategy for use as biopesticides in agro-systems. This will allow both the protection of crops against pathogens, with the consequent decrease in the use of synthetic fungicides and the negative effects (economic, environmental and health) associated with them. However, the wide use of lipopeptides in the agricultural, pharmaceutical, food sector, among others, depends on the development of chemical and analytical processes for the extraction, identification, quantification and production of said bioactive compounds, efficiently and economically.

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