Chapter 11

Production of Lipopeptides by Fermentation Processes: Endophytic Bacteria, Fermentation Strategies and Easy Methods for Bacterial Selection

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Abstract

Lipopeptides constitute an important class of microbial secondary metabolites. Some lipopeptides have potent therapeutic activities such as antibacterial, antiviral, antifungal, antitumor and immunomodulator. Surfactin, iturin, fengycin, lichenysin and bacillomycin D from *Bacillus* species, daptomycin from *Streptomyces roseosporus* and rhamnolipids from *Pseudomonas aeruginosa* are among the most studied lipopeptides. These molecules are good candidates to replace those antibiotics and antifungals with no effect on pathogenic microorganisms. Microbial lipopeptides are produced via fermentation processes by bacteria, yeast and actinomycetes either on water miscible and immiscible substrates. However, the major bottlenecks in lipopeptide production are yield increase and cost reduction. Improving the bioindustrial production processes relies on many issues such as selecting hyperproducing strains and the appropriate extraction techniques; purification and identification by Polymerase Chain Reaction(PCR), High Performance Liquid Chromatography-Mass Spectrometry(HPLC-MS), Matrix Assisted Laser Desorption Ionization-Time of Flight-Mass Spectrometry(MALDI-TOF-MS); the use of cheap raw materials and the optimization of medium-culture conditions. The purpose of this chapter is to orient the reader on the key elements in this field, including the selection of analytical strategies to get a good microbial strain as well as to show some examples of liquid and solid-state low-cost fermentation processes. Last, we introduce endophytic bacteria as lipopeptide-producer candidates.

Keywords: endophytic bacteria, fermentation, lipopeptide-producers, microbial lipopeptides, quorum sensing

1. Introduction

In recent years, the production of microbial lipopeptides (LPs) has been widely studied for their biotechnological application in several areas including pharmaceutical industry, food preservation and agriculture. Lipopeptides are characterized for their highly structured diversity and their ability to decrease the surface and interfacial tension. Structurally, they consist of a hydrophilic peptide and a hydrophobic fatty acid acyl chain. The number of amino acids generally varies from 7 to 25, whereas the length of the fatty acid chains varies from 13 to 17 carbons. One strain is able to produce several isoforms of the same polypeptide. *Bacillus*- and *Paenibacillus*-related lipopeptides (firmicutes) and *Pseudomonas*-related lipopeptides (Proteobacteria) are the most studied [1,2]. Besides, LPs can also be produced by *Streptomyces* [3,4] and fungal strains [5]. The LPs are highly variable and their structural analogues results from frequent amino acid substitutions. Among the most documented LPs produced by *Bacillus* strains are surfactin, iturin, fengycin and lichenysin. On the other hand, those produced by *Pseudomonas* strains are viscosin, tensin, arthrofactin, massetolid, pseudodesmin, xantholysin, pseudofactin and syringomicin. These lipopeptides as many others, are good candidates to replace those antibiotics and antifungals with no effect on the control of pathogenic microorganisms.

Lipopeptide surfactants are naturally produced as mixtures of several macromolecules belonging to the same family or class. The nutritional parameters can influence the nature of the produced LPs [6]. However, the major limitations on their production are the production costs and yields. A wide range of carbon sources and culture conditions have been reported in order to increase the production of iturins, surfactins and fengycins. Nowadays, a variety of cheap raw materials have been used in their production: rice bran, soybean, potato peels, molasses, etc. In addition, it has been demonstrated that divalent cations have an important influence on LP production, mainly Mn^{2+} and Fe^{2+}. The manganese addition to the medium culture increased yield rates from 0.33 to 2.6 g/L [7]. Furthermore, the presence of $ZnSO_4$, $FeCl_3$ and $MnSO_4$ increased surfactin production in *Bacillus subtilis* [8]. In this context, we will highlight the key parameters for the maximization of LPs production and to development future strategies for optimizing liquid and solid-state fermentations (SSFs). Both fermentation types are important on industrial scale production processes.

Another important issue to address here relates to the microbial-producing strains. The genetic load of the microorganisms is a determining factor on LPs production yields, since the capacity to generate a metabolite is controlled by genes. There is a need for hyperproducer strains. But how can we recognize these over producing microbes? Lipopeptides production can be detected by: (i) culture dependent methods; (ii) methods relying on surface analysis and emulsifying activity; (iii) cell surface hydrophobicity and (iv) chemical identification [9]. An optimal and widely accepted study must combine the genetic identification and the structural

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and genetic analysis of the produced LPs by the particular isolate. This methodology assures the phenotypic and genotypic features of the microbial isolates for LPs production. Currently, the PCR and gene sequencing are quick tools for screening and identification of microbial-producers, as well as to identify the genes involved in the LPs synthesis. Also, the isolation and identification techniques such as liquid chromatography coupled to mass spectrometry (LC-MS) and more recently the MALDI-TOF mass spectrometry, have been considered as the fastest and most efficient tools for identification of LPs in mixtures and peptide sequencing, respectively. These techniques are also useful to identify the most novel LPs and even the small compositional changes in the sequence of amino acids that will determine its properties. There is no doubt, that these tools will increase and incentive the development of this field in fermentation processes.

In our lab, we are particularly interested in bacterial endophytes. By definition, an endophyte is a bacteria or fungus that lives in the internal tissues of plants without disease manifestation. Endophytes are ubiquitous to virtually all-terrestrial plants. With the increasing appreciation of studies that unravel the mutualistic interactions between plant and microbes, functions of endophytes are gaining value, so these microorganisms have become the target of biotechnological developments for biological control of plant pathogens (fungi and bacteria). We have evidence that a large group of endophytic bacteria have the ability to eradicate their competitors (pathogens) from the niche using LPs. In this chapter, we are interested in discussing endophytic microorganisms known as lipopeptide-producers beyond the genus *Bacillus*. We believe this information will be of value for alternative research in agricultural microbiology as well as for the production of antimicrobial molecules. However, given that some endophytic bacteria have a closer relationship with human pathogens, the application on commercial crops as biopesticides is strictly regulated. This last point impacts negatively the use endophytic microbes as tools for disease control and the development of new bioinoculants for agriculture; therefore, such LPs must be produced by fermentation.

Finally, it must be considered that the production of biosurfactants is associated with the physiological status of the bacteria, where quorum sensing (QS) is probably a condition. Quorum sensing is perhaps an overlooked variable in fermentation processes. In this chapter, we try to explain how this phenomenon and other conditions can alter the performance of LP production.

2. Lipopeptides: classes, microbial producers, fermentation processes and downstream processes

2.1. Classes of lipopeptides and their applications

The production of LPs in their active form requires transcriptional induction, translation and post-translational modifications. The main machinery for their synthesis is multi-modular and consists of non-ribosomal peptide synthetases (NRPSs) [10]. Synthetases are organized on modules; each module permits the incorporation of a specific amino acid, subsequent condensation, termination and cyclization of the peptide chain. The synthesized peptides contain

D-amino acids, β-amino acids and hydroxyl- or *N*-methylated amino acids. The integrated system introduces heterogeneity among LPs. The peptide moiety is inactive until it is coupled to a fatty acyl chain. The lipid aliphatic chain, of variable length, fuses with the N-terminal residue of the peptide chain, and then the bioactive LP is generated. After the biosynthesis of the LPs is finished, the molecule is modified by glycosylation or halogenation by specific enzymes associated to the synthetases [11].

	Lipopeptides	***Microbial-producers***	**Activity roles**
Surfactin family	Surfactin linchenysin pumilacidin WH1 fungin	*B. subtilis* *B. polyfermenticus* *B. megaterium* *B. licheniformis* *B. pumilus* *B. amyloliquefaciens*	-Enhanced oil recovery Antibacterial Antiviral Antimycoplasma Antitumoral Anticoagulant Enzyme inhibition
Iturin family	Iturin Bacillomycins Mycosubtilin Subtulene (contains a unique Iso C15-long chain β -amino acid)	*B. subtilis* *B. megaterium*	-Antifungal -Biopesticides
Fengycin family	Fengycin Plipastatin Agrastatin1	*B. subtilis* *B. thuringiensis* *B. circulans* *B. megaterium*	-Strong fungitoxic agent against filamentous fungi -Immunomodulating activities
Pseudomonas sp. Lipopeptides	Viscosin Massetolide Entolysin	*Pseudomonas sp*	-Antibacterial and Antifungal activity against; *Mycobacterium tuberculosis*, Gram positive bacteria, *G. candidum* and *R. pilimanae*
Streptomyces sp. Lipopeptides	Daptomycin	*Streptomyces roseosporus*	-Broad spectrum activity against *staphylococci* (MRSA), beta-hemolytic *Streptococcus spp., Pneumococci, Clostridium spp., and Enterococci sp.* –MRSA *Staphylococcus aureus* -Antiparasitic -Immunosuppressor

Table 1. Important and the most studied lipopeptides from *Bacillus*, *Pseudomonas* and *Streptomyces* and their activity roles.

Many bacteria and some fungi produce LPs, which have several roles including activity against bacteria, fungi, virus and more recently, it has been discovered their antitumor activity. Lipopeptides are also are involved in bacterial motility, in the swarming behavior and in the attachment to surfaces [1]. On the other hand, the extensive use of chemicals to control pathogens (bacteria and fungi) has modified the behavior of these microorganisms in humans and plants. The growing drug resistance, in these pathogens, urges for alternative antimicrobial molecules for clinical and crop protection, as well as for food preservation. As we mentioned above, LPs can be cyclic or linear based on the topology of the peptide chain. Here, we

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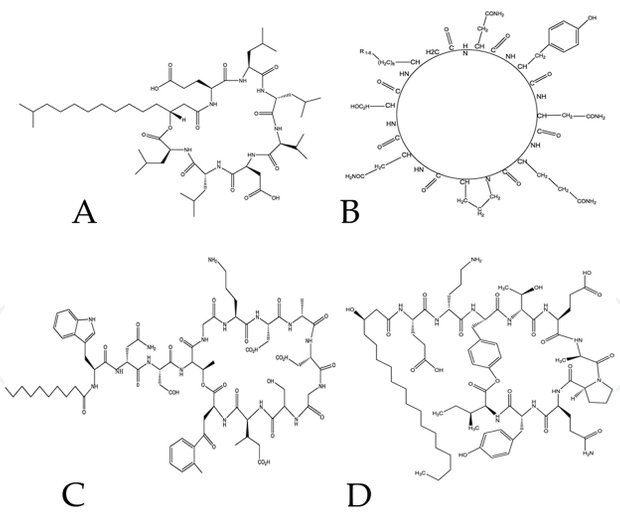

Figure 1. Pharmaceutically and economically important lipopeptides. Structures of representative member of lipopeptide synthesized by *Bacillus* and *Streptomyces*. (A) Daptomycin, (B) surfactin, (C) iturin and (D) fengycin.

present an updated overview on the bioactive LPs and their uses, being the cyclic lipopeptides the most biologically relevant with proved activity and more market applications in several fields. The characteristics of the LPs are discussed below, and their properties, structures and uses have been summarized in **Table 1** and **Figure 1**.

Daptomycin. This structure is a cyclic decanoyl lipid chain attached to 13 amino acids (a 10-member macrolactone and three exocyclic residues) peptide. It is produced by *Streptomyces roseosporus*, a Gram-positive bacterium. It has potent antimicrobial properties and it has been clinically approved for its use as antibiotic since 2003. It is marketed under the tradename Cubicin. The mode of action of daptomycin is still unclear, but two hypotheses have been proposed, the first one states the inhibition of lipoteichoic acid synthesis (proteoglycan component of the cell wall of Gram-positive bacteria); the second states the disruption of bacterial membrane potential (depolarization) via pore formation and its calcium ion dependence. Concomitantly, the bacterial cell loses the ability to accumulate amino acid substrates while leaving glucose uptake intact [12,13]. It has been successfully used to control skin infections, endocarditis, osteomyelitis and soft-tissue infections [14]. The cost of daptomycin in the market is approximately €125/day at dose of 6 mg/kg/day.

Surfactin. Surfactins constitute a major class of antibiotic LPs produced by *Bacillus* spp. They are highly active biosurfactants able to reduce the surface tension of water to 27 mN m^{-1} at 20 μmol. This group consist of a heptapeptide bonded to a C_{13}–C_{15} fatty acyl chain [15]. Surfactins are able to permeate the lipid membranes as dimer and form ion channels in planar lipid bilayer membranes. These compounds are effective against Gram-positive and Gram-negative bacteria and also have antimycoplasma, antiviral and antitumor activity and suppress inflammatory responses through inhibition of phospholipase A2 [16,17]. Surfactin also inhibits phytopathogens such as *Pseudomonas syringae*, *Xanthomonas axonopodis*, *Sclerotinia sclerotium*, *Botrytis cinerea*, *Colletotrichum gloeosporioides* and stimulates plant defense [18,19]. Since surfactins have hemolytic activity their medical applications are limited.

Iturins. Iturin is an antifungal cyclic lipopeptide produced by *Bacillus* spp. These amphiphilic compounds are characterized by a peptide ring of seven amino acid residues including an invariable D-Tyr2, with the constant chiral sequence LDDLLDL closed by a C14–C17 aliphatic β-amino acid. Iturins have a high polymorphism due to amino acid variations. These variants including iturin A, iturin C, iturin D, iturin E, Bacillomycin D, Bacillomycin F, Bacillomycin Lc, Mojavensin A and mycosubtilin [20]. Iturin A has been shown to form potassium ion-conducting channels in lipid bilayers. Iturins can act as biocontrol agents of plant pathogens [14]. They exert their fungicidal action by interacting with sterol components in the fungal membrane. Mojavensin A, a new member of the family, is cytotoxic [21]. From a clinical perspective, a disadvantage associated with iturins is their haemolytic activity.

Fengycins. This class of cyclic lipopeptides includes fengycins and plispastatins produced by some *Bacillus* and *Paenibacillus* strains. Fengicins are decapetides acylated with a β-hydroxylipid tail (C14–C18) and cyclized between the phenol side chain of Tyr 3 and the C-terminus. They act on plasma membrane of fungal cells and have been suggested for agriculture. They have antitumor activity because of the production of reactive oxygen species and mitochondria-dependent apoptosis [22]. They are good candidates for medical applications due their milder haemolytic activity.

Pseudofactin I and II. These compounds are cyclic octapeptides bonded to palmitic acid produced by *Pseudomonas fluorescens* BD5. The C-terminal of the carboxylic group forms a lactone with the –OH of threonine. Their emulsification activity and stability are greater in comparison to other synthetic surfactants, thus have a great potential for bioremediation or biomedicine. For example, Pseudofactin II exerts cytotoxicity in human melanoma [23].

Viscosin is obtained from *Pseudomonas fluorescens*. It has antibiotic activity, is highly surface active and is able to inhibit the migration of cancer cells. In *Pseudomonas*, this LP protects it from protozoan predation. Viscosin increases the efficiency of surface spreading over plant roots and protects germinating seedlings in soil infected with plant pathogen [24].

Linear cationic lipopeptides. Limited research has been performed on this small group. This is surprising as they have the potential to be more accessible than cyclic lipopeptides. This group includes saltavalin (which was named because contains serine, alanine, leucine, threonine, valine and 2,4-diaminobutyric acid, isolated from *P. polymyxa*), jolipeptin (*B. polymyxa*), cerexins (*B. cereus*, *B. mycoides*), tridecaptins (*P. terrae*). All exhibit antibacterial activity against Gram-negative and Gram-positive bacteria.

2.2. Endophytic bacteria as lipopeptide-producers

Due to the nature of the endophytic life style, endophytic microbes establish a long-lasting stable relationship with the plant. In this symbiotic association, the plant provides nutrients and shelter for the microbes and, in turn, the endophyte benefits plants by imparting biotic and abiotic stress tolerance and promoting its growth. Some endophytes are known to produce anti-pest compounds. These bioactive secondary metabolites can be either directly involved in antibiosis and/or triggering induced systemic resistance (ISR). There have been published some reports of the production of LPs by endophytic bacteria that may explain the antifungal

or antibacterial activity on plant pathogens. Recently, Gond et al. reported an endophytic *Bacillus* that produces antifungal LPs and host defense gene expression in maize [25]. Understanding the mechanism of biological control helps to manipulate the environment to create conditions for better biocontrol. Nowadays, in Mexico, some groups are developing research focused in field applications of LPs rather than bacteria, since many of the bacteria belong to genera related to human pathogens. In unpublished studies from our group, we have found strains of bacteria with the ability to produce LPs isolated from plants, including agave, banana and maize (Beltran-Gracia, manuscript in preparation). In **Table 2**, we list some of endophytic strains reported to be lipopeptide-producers.

Endophytic lipopeptide-producers	**Secreted lipopeptides**		
B. subtilis	Surfactin	Iturin	Linchenysin
	Pumilacidin	Bacillomycins	Mycosubtilin
	Fengycin	Plipastatin	
B. amyloliquefaciens	Surfactin	Linchenysin	Pumilacidin
	Fengycin		
B. megaterium	Surfactin	Linchenysin	Pumilacidin
	Iturin	Bacillomycins	Mycosubtilin
	Subtulene	Fengycin	Plipastatin
B. circulans	Fengycin	Plipastatin	Iturin
B. tequilensis	Surfactin	Iturin	Fengycin
B. polymyxa	Polymyxin		
B. macerans	Surfactin	Iturin	Fengycin
Streptomyces sp.	Daptomycin		
Pseudomonas sp.	Viscosin	Massetolide	Entolysin

Table 2. Endophytic bacteria reported as lipopeptide-producers.

2.3. Lipopeptide production by fermentation process: culture conditions and operational conditions

In order to incorporate LPs into industrial processes and for medical, pharmaceutical and agricultural uses, it is required their production by fermentation and their posterior downstream. It is clear that one of the main limitations for commercial applications of LPs are the high production costs and the low yield. To overcome these barriers, many efforts have been focused in improving the fermentation process, which represents a fundamental stage in the global production. Lipopeptides have been reported as growth-associated metabolites. In contrast to other bacterial secondary metabolites, production of LPs is induced when the cells have exhausted one or more essential nutrients, in example, surfactin production is induced

in actively growing cells during the transition from exponential to stationary phase (SP); fengycin synthesis is related to the early SP, and iturins only accumulate in the later SP [1].

The production of LPs can be achieved by liquid fermentation (LF) or solid-state fermentation (SSF) and now, both methods have been proposed for scale up their industrial production. The LF is an advantageous and typical process used for LPs production in controlled bioreactors, while SSF is still in evolution but has gained attention owing its priority to LF, including lower investment for production, less time and higher secondary metabolite yields.

A critical factor into industrial LP production is media optimization. In fact, the nature of the carbon substrate, N, P, Na, Mg, Fe, Zn and Mn ions concentration in the medium, have been shown to influence enormously the nature and quantity of the LP produced by several bacterial strains. An orderly and planned statistical procedure to screen the effect of each component of the media is very useful. For example, a Plackett-Burman procedure was applied to find that glucose, K_2HPO_4, and urea concentrations had the most influence into LPs production by *Bacillus subtilis* of 11 tested variables (glucose, urea, ammonium sulfate, NaCl, $MgSO_4$, KH_2PO_4, K_2HPO_4, $MnSO_4$, $FeSO_4$, $ZnSO_4$). After, a Central Composite Design was conducted to optimize the three selected factors, finding a maximum biosurfactant concentration of 3.1 g/L when using 15 g/L glucose, 6 g/L urea and 1 g/L K_2HPO_4, keeping the other parameters at their minimum values [26]. A similar statistical procedure was also applied to determine the effect of sucrose, ammonium nitrate, NaH_2PO_4, K_2HPO_4, $MgSO_4$, $MnCl_2$, extract yeast as components of culture media to growth *Bacillus amyloliquefaciens*, using the Plackett-Burman design in the production of C15-surfactin, indicated a significant effect of sucrose, ammonium nitrate and NaH_2PO_4. The optimum values of the tested variables were 21.17 g/L sucrose, 2.50 g/L ammonium nitrate and 11.56 g/L NaH_2PO_4 with a production of 134.2 mg/L LP [27]. In a third case, a five-level four-factor Central Composite Design was employed to determine the maximum LP yield by *Bacillus subtilis* testing sucrose, ammonium chloride, ferrous sulphate and zinc sulphate. Optimum fermentation components were 22.431 g/L of sucrose, 2.781 g/L of ammonium chloride, 6.7879 mM of $FeSO_4$ and 0.0377 mM of $ZnSO_4$ to produce 1.712 g/L of LP. Only the ammonium chloride had no significant effect [28].

2.3.1. Carbon sources to optimize lipopeptide production

It is clear the importance of carbon source in any fermentation process due to its impact in the bacterial metabolism as well as in production costs. The nature and quantity of the carbon source are the most important factors that would affect LPs production. Structural and compositional diversity of LPs is substrate dependent. For example, *Bacillus amyloliquefaciens* was grown in a minimal salt medium with different carbon sources (sucrose, dextrose, maltose, lactose, glycerol and sorbitol) where a C:N ratio remained constant at 15.55. The surfactin, iturin and fengycin were detected when dextrose, sucrose and glycerol were used as carbon source. However, in the presence of maltose, lactose and sorbitol only iturin was produced. Also, these carbon sources significantly influenced the antifungal activity of the molecules. Those bacteria grown in media supplemented with dextrose or sucrose produced LPs with the higher antifungal activity. The maximum biosurfactant activity was observed when the growing minimal salt medium was supplemented with sucrose [29]. In a similar study, it was

reported that among several carbon sources: glucose, sucrose, galactose, maltose, sucrose, glycerol, mannitol, soluble starch and dextrin, evaluated for C15-surfactin production, sucrose was the best carbon source [27].

2.3.2. Nitrogen sources

Several inorganic nitrogen compounds have been tested in LP production trials, i.e. ammonium nitrate, ammonium sulphate, sodium nitrate, urea and glutamic sodium. And looking for cheaper raw materials, complex compounds such as soybean flour, peptone and casein acid hydrolysate have been assayed too [27]. When using organic nitrogen sources it was observed that tryptone enhances the lipopeptide production because it contains several homologous L-amino acids to those found in LPs [30]. A similar behavior was reported in the modified Landy medium, where L-glutamic acid was replaced with various L-α-amino acids at the same concentration (5 g/L). Cottonseed-derived medium (Pharmamedia) proved to be a suitable substrate for the production of 220 mg/L of surfactin from *Bacillus subtilis*; this medium is suitable to achieve high production yields at low cost, which in turn makes it profitable for large scale usage. Moreover, supplementing Pharmamedia with Fe^{2+} (4.0 mM) and sucrose (2 g/L) leads to a maximum production of about (300 mg/L) [31]. Other interesting nitrogen source is rapeseed meal, a low-cost material that was used to synthetize iturin A by *Bacillus subtilis*. The maximum iturin A concentration was 0.60 g/L after 70 h of incubation, which was 20% and 8.0 times higher than that achieved with peptone and ammonium nitrate media, respectively [32].

2.3.3. Divalent ions

To optimize the trace element composition of culture media the use of statistical experimental designs is preferred. Such is the case, where was doubled surfactin concentration when was applied the statistical method Taguchi to determine cation effect of culture medium [33]. The role of Fe^{2+} in the synthesis of LPs is crucial; there are some reports where the supplementation of this cation enhanced the yield of biosurfactants [31,33]. The addition of Fe^{2+} into fermentation medium was utilized to optimize surfactin production from *Bacillus subtilis*, reaching yields up to 3 g/L into minimal salt medium; the optimal Fe^{2+} dosage (4.0 mM) leading to 8-fold and 10-fold increments in cell concentration and surfactin yield, respectively, as compared to those media without Fe^{2+} [34]. *Bacillus amyloliquefaciens* in a culture medium supplemented with 0.2 mM of iron was able to produce 92.78 mg/L of iturin after 5 days with no pH control in the culture. Moreover, if the starting pH at 6.64 and 0.2 mM of ferrous sulphate, an iturin A production of 121.28 mg/L was obtained [35].

2.3.4. Operational conditions

The pH, temperature, dissolved oxygen concentration and degree of aeration affect cellular growth, and consequently, biosurfactant production. Optimal operational conditions vary from strain to strain, and the better growing condition for each particular strain must be determined experimentally. For example, surfactin synthesis by *Bacillus subtilis* can be achieved at temperatures ranging from 25 to 37°C; the optimal temperature for the surfactin produc-

tion by *Bacillus subtilis* DSM 3256 was 37.4°C. In contrast, for thermophilic *Bacillus* spp. surfactins were produced at temperatures above 40°C without detriment on their activity [36]. Regarding pH, a greater LP activity was observed when the pH was adjusted to between 3.0 and 8.0.

Statistical tools had been used to optimize LP biosynthesis considering operational conditions. The response surface methodology has been used to determine the maximum LP production varying the temperature, initial pH and culture cycle. Another important condition to be controlled is the dissolved oxygen. The oxygen acted at different levels, suggesting a complex system for regulating the synthesis of LP in B. *subtilis* ATCC6633. So, the oxygen transfer is one of the critical parameters for process optimization and scaling-up of production of surfactin [36]. Varying the oxygen transfer conditions, the synthesis could be oriented to mixed production or to surfactin monoproduction. The fraction of surfactin towards total LPs produced and the maximal surfactin production both increased with $k_L a$ increase (surfactin concentration about 2 g/L at $k_L a$ = 0.04–0.08 s^{-1}), while the maximal fengycin production (fengycin concentration about 0.3 g/L) was obtained at moderate oxygen supply ($k_L a = 0.01\ s^{-1}$). The production of LP represents a challenge due to its surface properties. Foam causes a severe decrease of oxygen transfer. A significant decrease of $k_L a$ (up to 27%) was measured during fermentation process reduces LP biosynthesis [37].

Classical bioreactors aerated by gas bubbling are not suitable for production of LP biosurfactants due to excessive foaming. The use of antifoam agents is not appropriate because it can affect the bacterial physiology and downstream processing. The alternatives are bioreactor with foam collector, rotating discs biofilm reactor [38], bubbleless membrane aerated bioreactor [39] and three-phase inverse fluidized bed bioreactor [40]. Another alternative to foam control is the use of a strictly anaerobic bioreactor cultivation to produce surfactin. Most interesting, the product yields exceeded classical aerobic fermentations, in which foam fractionation was applied. Additionally, values for specific production rate surfactin (0.005 g/(g h)) and product yield per consumed substrate ($Y_{P/S} = 0.033$ g/g) surpass results of comparable foam-free processes [41]. The bioreactor design is still a challenge to get better productivities in industrial processes; the LPs synthesis is not an exception.

2.4. Solid-state fermentation (SSF)

Solid-state fermentation (SSF) is an alternative technology for the production of high-value molecules. The SSF could be an alternative method for the LPs production. The SSF uses agro-industrial wastes as substrates, which contributes to reduce the production costs. The productivity of LPs synthetized by SSF depends on initial moisture content, incubation temperature, fermentation time, substrates used and supplementary nutrients such as mineral salts. The temperature is an important parameter for both bacterial growth and LP production. Within a range from 25 to 40°C, the optimal temperature for growth was found to be 30°C, but the biosynthesis of LPs is favored at 37°C. At the beginning, in the firsts 24 h, temperature should be maintained at 30°C, then shifted up to 37°C to enhance LP production [42].

The selection of raw materials to formulate a culture medium, as the previously described, requires experimental tests. Savings in time and resources can be achieved using statistical

methods, which help in optimizing components and concentrations in the formulation of a culture medium. The optimal composition of culture medium in solid-state fermentation to get the highest LP production had been determined using a 'Central Composite Design'. First, a screen to select the major solid substrates was performed, where rapeseed meal, corn flour, soybean flour, bean cake, wheat bran, rice hull and rice straw were considered as candidates to support bacterial growth and biosurfactant production. For the election, a quantity of each solid substrate was supplemented with 1.0 ml of mineral solution with initial pH 7.5 and moisture content 55%. To increase the porosity of the substrates and improve its availability, each of them was mixed with an inert substrate (perlite, vermiculite, beads). After that, both easily digestible carbon sources (glucose, sucrose, starch, L-glutamic, maltose, glycerol, D-galactose) and nitrogen sources (tryptone, peptone, yeast extract, urea, NH_4NO_3, NH_4Cl, $(NH_4)_2SO_4$ at 2% (w/w) were added. The substrates selected to the optimization of the medium composition for LP production by 'Response Surface Methodology' were soybean flour, rice straw, starch and yeast extract. The optimal conditions were 1.79% starch and 1.91% yeast extract by employing 5.58 g soybean flour and 3.67 g rice straw as the solid substrate with initial pH 7.5, moisture content 55% and a 10% inoculum level at 30°C for 2 days. Under these conditions, the experimental yield of LPs reached 50.01 mg/gram of dry substrates [43]. SSF many times is compared with submerged fermentation. By this reason, a comparative study was performed to determine the compositions and properties of LP products purified and the transcription values of LP genes under submerged fermentation and SSF. Results revealed no significant differences in the polarity and structure of the two LP products. But, LP obtained by submerged fermentation possessed higher amino acid proportions, better emulsification activity and antagonistic activity than that from solid-state fermentation. For solid-state fermentation, the transcription accumulation levels of the LP synthetic genes srfA and sfp were higher than for submerged fermentation at the same stage. Transcripts for ituD and lpa-14 remained elevated for a longer period of time under solid-state fermentation conditions, accounting for differences in the production and fermentation periods between both fermentation techniques [44].

2.5. Downstream processing: isolation and purification of lipopeptides from fermentation process

Due to different applications of LPs, we need different levels of purity. Crude LPs can straightaway be used in bioremediation related applications, where the overall economy of the process is the most important concern. On the other hand, partially purified fractions (about 60–80% pure) can suit applications in microemulsion based nanoparticle synthesis, laundry and food industry. However, the requirement for ultrahigh pure product is indispensable, if the LPs are to be considered for pharmaceuticals and human healthcare [45]. As we mentioned before, the intense foaming produced during aerobic liquid fermentation is a big obstacle for the commercialization making their recovery and purification difficult. A great deal of monetary input will be required for purification, this account 60% of the total production cost. Different techniques have been developed to extract and purify LPs. Among the most used techniques to extract are acid precipitation (HCl 6N), solvent extraction (chloroform, ethyl acetate, dichloromethane or mixtures of chloroform-methanol), ammonium sulphate precipitation

with dialysis to remove small molecules and salts, and foam fractionation (utilized for continuous retention process and high purity). For purification normally have been used membrane ultrafiltration techniques, ionic exchange chromatography and adsorption-desorption on resins (XAD-4, XAD-7 HP, HP-2MG, HP-20) or activated carbon. The High Performance Liquid Chromatography(HPLC) (an excellent method for separation of this class of molecules) uses a reverse phase with C18 columns. The LPs separated can be detected using ultraviolet absorbance or diode arrays detectors and each peak separated is collected using a fraction collector for further analysis of their structure.

2.5.1. Example of techniques used for lipopeptide recovery, purification and identification

Membrane ultrafiltration. This technique serves essentially as an intermediate process for the recovery and purification of LPs. The separation of LPs by membrane filtration depends on their molecular aggregation behavior and on their ability to form micelles, since a process become economic feasible when a high MWCO (molecular weight cut-off) membranes were used. In general, the use of low MWCO membranes requires high maintenance due to low permeate fluxes through smaller pores that get easily plugged by monomers and progressive reduction in flux caused by the mechanism of concentration polarization [45].

A two-step ultrafiltration process using large pore size membranes (up to MWCO = 300 kDa) was investigated to separation of LPs aggregated in single and mixed solutions from fermentation culture. In single solutions of LP both surfactin and mycosubtilin formed micelles of different size depending on their concentration. However when the LPs were in the same solution, they formed mixed micelles of different size and probably conformation to that formed by the individual LPs, this prevents their separation according to size. An effective rejection in the first ultrafiltration step was achieved by membranes with MCWO= 10–100 kDa but poor rejection by the 300 KDa membrane [46]. The rejection is a measure of retention capacity of a membrane. However, some properties of LP micelles such as poor stability and non-uniform size distribution limit the use of readily scalable high MWCO membranes for the purification of LP, as smaller sized micelles and monomers can easily pass through the pores of these membranes. An addition of Ca^{2+} ions causes the structural transformation of surfactin monomers to larger micellar aggregates, showing excellent features such as compact structure, narrow size distribution, and improved stability [45].

Chromatographic technique for daptomycin. Daptomycin was purified from clarified fermentation broth using anion exchange chromatography and reverse phase chromatography. The anion resin was a highly cross-linked agarose with dextran surface extender. Daptomycin was eluted from the anion exchange column with a NaCl gradient from 0.2 to 1.0 M in water. The semi-purified daptomycin was then added to a reversed phase column and was washed with water containing 15% of alcohol. The reverse phase resin was a mono-sized, porous resin made of polystyrene and divinyl benzene (source: RPC 30). After, daptomycin was eluted with 40–70% of ethanol. Two reverse phase columns were involved to improve the purity of the final product at different pH. The first column was run at pH 7.5–8.0, while the second one was eluted at pH 3.0–3.1. The purified daptomycin is then filtered and lyophilized under standard conditions with at least 95% of purity [47].

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Resins. Macroporous adsorption resin (MAR) chromatography has been successfully used for separation of bioactive molecules on the basis of hydrophobic/hydrophilic interactions between solute and resin surface. MAR has been resolved problems related with low efficiency to separate LP mixtures into individual families employing a simple stepwise solvent gradient elution under optimal conditions. The adsorption and desorption of solutes on MARs depend upon the properties of the resins such as particle size, pore diameter, surface area and polarity. An example is the performance of a non-polar resin (HP-20) that combine features such as higher surface area, pore size and appropriate polarity, allowing it to have a superior adsorption capacity over other resins. Dual gradient MAR was applied to a cell free broth diluted until its total crude LP concentration was 3 g/L, then it was pumped at a flow rate of 1 ml/min into the column pre-packed with HP-20 resin (15 g) until breakthrough point. After one run of adsorption and desorption, the three LP families were successfully enriched in separate fractions and the recovery yields were 79.5% for iturin, 94.4% for fengycin and 89.4% for surfactin. Their purities in the enriched fractions were found to be 68.3, 77.6 and 91.6%, respectively. This process represents a basis for *in situ* recovery of LPs from the culture broth in continuous mode [48].

2.5.2. HLPC-MALDI-TOF

HPLC is an excellent method for the separation of individual LP separation. The most employed technique is reverse phase chromatography, due to this method can separate this metabolite based on its polarity. The separated products are detected by UV absorbance detection and each individual peak can be collected for further analysis of their structure. Also, use of a diode array detector is recommended. This detector can measure simultaneous wavelengths in a range of 200–600 nm, this means that we can detect LPs as they are eluted from the column in several wavelengths. For mobile phases reported, the methanol:water (80:20) is the most commonly employed, due this phase can elute several LPs as fengicins and iturins. Also, another mobile phase used is acetonitrile:water, as the methanol:water phase, this mobile phase can elute LPs as surfactins. The proportions for a acetonitrile:water can change depending on the LP that you want to separate. The typical column for LP's separation is a C-18 column, the length can vary from 150 to 250 mm, and this depends on the resolution and separation desired. In terms of particle size, 5 μm is the most adequate for the stationary phase. **Table 3A** shows some conditions of HPLC most widely used for separation as well as quantification of LPs derived from a fermentative process [49,50].

MALDI-TOF (matrix-assisted laser desorption/ionization-time of flight) is a mass spectrometry technique that allows the identification of intact compounds. Samples to be analyzed are mixed with a matrix and dried on a stainless steel plate, onto which a laser with various degrees of energy is fired to forming gaseous ions, which can be separated in a time of flight (TOF) analyzer and detected. Now, MALDI-TOF-MS has come to be regarded as a very fast and reliable tool for identification of LPs when compared to the conventional methods like culturing and purifying the LPs. The MALDI-TOF-MS for the identification of LPs has been previously reported [51, 52]. For LP identification, we can use CHCA (α-cyano-4-hydroxycinnamic acid), SA (sinapic acid) and DHB (2,5-dihydroxybenzoic acid). To the best of our

knowledge, DHB matrix is better than CHCA, because we obtain a good quality spectra with intensities above 2E4 and LP isomers can be observed. In **Table 3B** are shown relevant information of the mass range for specific LPs identification derived from MALDI-TOF analysis.

TECHNIQUE	*CONDITIONS*		*LIPOPEPTIDE SEPARATED*
(A) HPLC	COLUMN	C-18, 150mm–250mm length, size particle 5μm	ITURIN, FENGYCIN SURFACTIN AMPHISIN LOKISIN HODERSIN, TENSIN VICOSINAMIDE DAPTOMYCIN VISCONSIN MASSETOLIDE ENTOLYSIN
	MOBILE PHASE	Methanol: water (80:20), Acetonitile:water (80:20 and 40:60), acetonitrile: acetic acid (68:32)	
	RETENTION TIMES	3-10 min iturin 10-16 min fengycin 16-25 min surfactin 28.5 min amphisin 29 min lokisin 29.2 min hodersin 29.9 min tensin 31.6 min vicosinamide	
	DETECTORS	UV-VIS (205, 235, 278, 285nm), diode array.	
(B) MASS SPECTROMETRY	**PEAKS (*m*/*z*)**	**IDENTIFICATED LIPOPEPTIDE**	
	1001.42, 1029.42; 1016.56, 1030.58, 1044.59, 1058.61, 1072.62; 1030.64, 1044.65, **1058.66**, 1072.67	ITURIN A	
	1052.63,1094.45, 1122.47, 1136.55; **1052.64**, 1066.57; **1052.62**, 1066.60, 1080.59	BACILLOMYCIN	
	1435.58, 1449.63, **1463.68** , 1477.66; 1421.61, 1435.66, 1459.69, **1463.71**, 1477.72; 1421.75, 1435.76, 1449.77, **1463.79**, 1477.60,	FENGYCIN	

TECHNIQUE	*CONDITIONS*		*LIPOPEPTIDE SEPARATED*
	1491.81		
	1488.70, 1502.71, 1516.62; **1488.71**, 1502.72; **1488.79**, 1502.79	FENGYCIN (POTASSIUM ADDUCT)	
	1074.58, 1088.57, 1102.55	BACILLOMYCIN (SODIUM ADDUCT)	
	1485.70 , 1499.71, 1513.71; 1471.75, **1485.76**, 1499.77, 1513.79	FENGYCIN (SODIUM ADDUCT)	
	878.47, 892.41, **906.50**	KURSTAKINS	
	1202.75, 1188.73, 1143.70, **1129.68**, 1168.77, 1154.75	POLYMYXINS	
	1421.75, 1407.73, 1393.72, **1379.70**	BACITRACINS	

Table 3. HPLC strategies and conditions (A) used for lipopeptides separation from extracts of culture filtrate and (B) ranges of m/z of typical peaks obtained by MALDI-TOF analysis. In bold, the reference peaks for lipopeptide identification.

2.6. Discovery of endophyte-producers of lipopeptides: combining molecular biology and chromatographic- mass spectrometry methods

To search for endophytic bacteria in nature that can produce LPs, researchers must optimize their screening and identification times. Actually, the strategies for the selection of endophytic microorganisms as candidates for biocontrol or lipopeptide-producers combines antifungal-antibacterial screenings, a molecular analysis of the genes involved in the LP synthesis and then an analysis of the extracts obtained from the culture medium by HPLC and mass spectrometry.

In practice, after endophyte isolation, we identify the purified strain by MALDI-TOF and confirm its identity by 16s rDNA sequencing. Once identified, we select the strain as a function of its antagonistic ability against plant pathogenic strains. For screening antifungal activity of the isolates, we use a dual culture test against fungal strains collected from plants and previously identified. Normally, the plates are incubated for 3–5 days to observe inhibition of the fungal mycelium (**Figure 2**). For screening the presence of LP product biosynthetic gene clusters present in the antifungal isolate, PCR-based screening methods are used for different genes involved. In **Table 4**, we show the summarized sequences and methods for PCR analysis most used for some lipopeptide-genes, then each amplification product is analyzed by agarose gel electrophoresis to compare the predicted base-pair number (**Figure 3**). The sequence data

obtained must be analyzed by BLAST and detailed *in silico* phylogenetic analysis to confirm the PCR product.

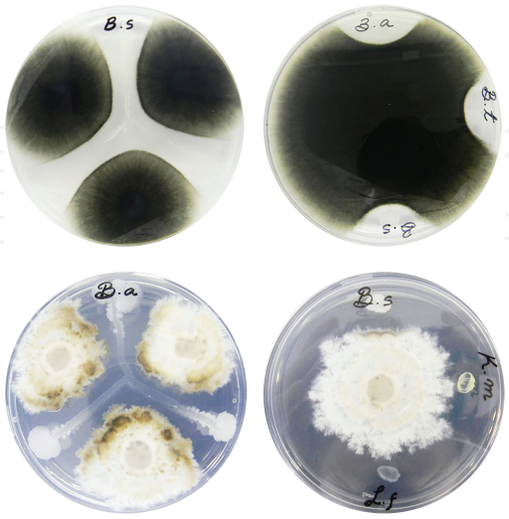

Figure 2. Antagonism of endophytic *Bacillus subtilis (Bs), B. amyloliquefaciens (Ba) and B. tequilensis (Bt), Kocuria marina (Km)* and *Lysinibacillus fusiformis (Lf)* isolated from agave, banana and maize against *Rhizotocnia* sp. and Colletotrichum sp. incubated by 5 days. It is noted that *Bacillus* strains used are the only ones that have antifungal activity, so the other strains should be discarded for identification analysis.

LIPOPEPTIDES	GENES	PRIMERS	PRIMERS SECUENCES (5′–3′)	AT*	PCR++
Iturin	ituD	ITUD-F1	TTGAAYGTCAGYGCSCCTTT	55	482
	ituC	ITUD-R1	TGCGMAAATAATGGSGTCGT	55	594
	ituA	ITUC-F1	CCCCCTCGGTCAAGTGAATA	55	647
		ITUC-R1	TTGGTTAAGCCCTGATGCTC		
		ITUD1F	GATGCGATCTCCTTGGATGT		
		ITUD1R	ATCGTCATGTGCTGCTTGAG		
Surfactin	srfA	SRFA-F1	AGAGCACATTGAGCGTTACAAA	55	626
	srfP	SRFA-R1	CAGCATCTCGTTCAACTTTCAC	52	675
	srf/Ich	SFP-F1	ATGAAGATTTACGGAATTTA	43	428
		SFP-R1	TTATAAAAGCTCTTCGTACG		
		As1-F	CGCGGMTACCGVATYGAGC		
		Ts2-R	ATBCCTTTBTWDGAATGTCCGCC		
Mycosubtilin	Myc/itu	Am1-F	CAKCARGTSAAAATYCGMG CCDASATCAAARAADTTATC	45	419
		Tm1-R			
Fengycin	fen	Af2-F	GAATAYMTCGGMCGTMTKGA GCTTTWADKGAATSBCCGCC	45	452
		Tf1-R			
Piplastin	pps	Ap1-F	AGMCAGCKSGCMASATCMCC	58	1029
		Tp1-R	GCKATWWTGAARRCCGGCGG		

*AT: ANNEALING TEMPERATURE (°C).
+PCR: PCR PRODUCT SIZE EXPECTED (bp).

Table 4. PCR primers commonly used for amplification of lipopeptide genes.

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The crude extracts obtained from culture media must be subject to HPLC separation and their collection for mass identification. The chromatographic profile of acidic methanol extracts and MALDI-TOF spectra of LPs from endophytic *Bacillus amyloliquefaciens* and *B. tequilensis shown* the presence of LPs groups iturins, fengycins and surfactin (**Figure 3**). Mass ranges (*m*/*z*) are 1001.42–1072 for iturin, 1471–75–1513.79, 1488.70–1516.62 for K and Na adducts of fengycin respectively and 1000–1100 for surfactin.

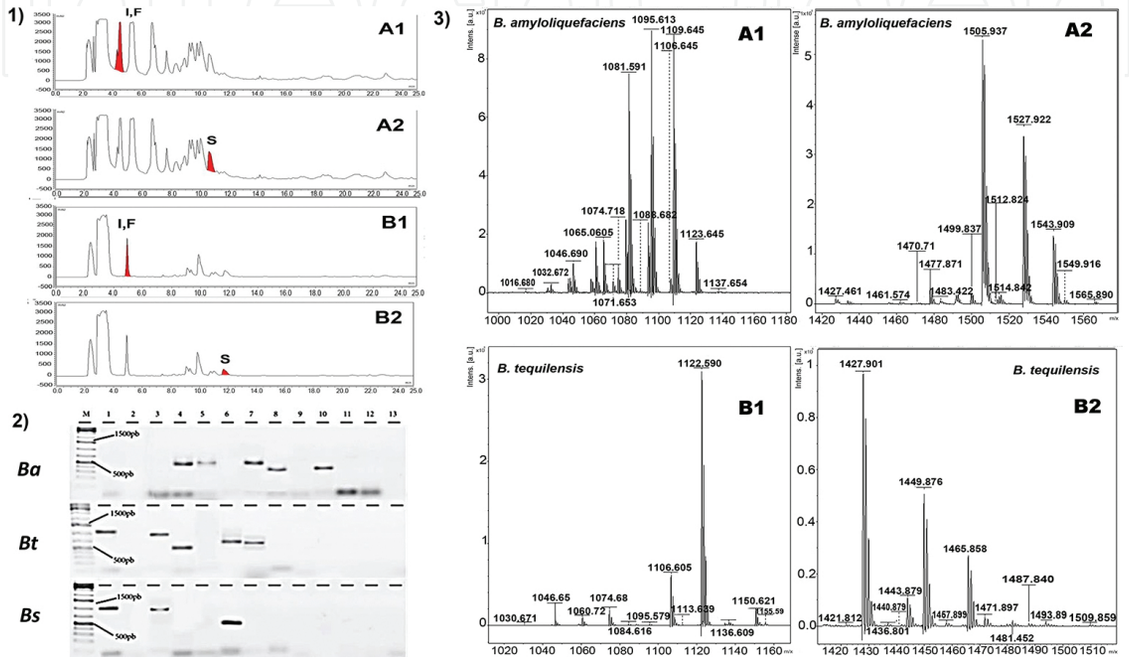

Figure 3. Agarose gel electrophoresis of PCR products of Bacillus endophytes (Below left) and separation and identification of the antifungal lipopeptides from acidic methanol extract by using reversed-phase HPLC(Above left) and MALDI-TOF MS analysis (Right) Section 2: PCR detection of lipopeptide and biosynthesis genes from Bacillus amyloliquefaciens (A), B. tequilensis (B), B. subtilis (C). M DNA ladder, Lane 1 Subtilosin, Lane 2 Sublancin, Lane 3 Plipastatin, Lane 4 Iturin D, Lane 5 Iturin C, Lane 6 Iturin A, lane 7 Surfactin A, Lane 8 Surfactin F, Lane 9 Mycosubtilin, Lane 10 Fengycin, lane 11 Subtilin A Lane 12 Ericin and Lane 13 Surfactin P. Chromatogram profile and MALDI-TOF mass spectrum of B. *amyloliquefaciens* (A) and B. *tequilensis* (B). The chromatograms were obtained under the following conditions: 0-3min: 45%-50% acetonitrile; 3-8min: 50%-80% acetonitrile; 8-25min: 80-100% acetonitrile, temperature of 38°C and a C-18 column (5μm particle size, 250 mm). Mass spectra were obtained with a RP 700-3500 Da, DHB as matrix. In section 1 (**A1** and **B1**), we can observe the mass spectrum of the HPLC fraction collected (within 4 min for A1 and 5 min for **B1**) that represent iturin (Section 3: with mass of 1016.68m/z and surfactin (1065.06 m/z) indicated that both strains produce this lipopeptides. In **A2** and **B2** we observe fengycin, in both cases the mass spectrum contains the representative mass of fengycin (1477 m/z) in section 3.

2.7. Quorum sensing in fermentative processes and lipopeptide production

Quorum sensing (QS) is a form of cell-cell communication by which bacteria communicate by secreting signaling molecules called autoinducers that help regulate gene expression. The QS molecule as N-acylhomoserine lactones in Gram-negatives or AIP in Gram-positives regulates different bacterial functions such as antibiotic biosynthesis, production of virulence factor, bacterial swarming, sporulation, competence and transition to the stationary growth phase.

How are LP production and quorum sensing associated? Surfactin a LP widely mentioned in this chapter book was proposed as a quorum-sensing molecule that activates the process of sporulation and production of biofilm [53]. For regulation of surfactin production by a cell density-responsive mechanism *B. subtilis* utilized a peptide pheromone Com X. Com X accumulates in the growth medium. So, the QS control the *srf* operon expression via Com X. However, few studies have been reported relating QS with fermentative processes. The production of putisolvins, also cyclic lipopeptides, in *Pseudomonas putida* occur at the end of the exponential growth phase, which indicates that the production of putisolvins is mediated through a quorum sensing-mechanism [54,55]. Another example that links QS with LP production is rhamnolipid a glycolipid biosurfactant produced also by *Pseudomonas spp*. The *rhl* quorum sensing system in *P. aeruginosa* regulates the production of rhamnolipid type biosurfactants. RhlA, a rhamnosyltransferase, catalyses the synthesis of fatty acid dimers that subsequently serve as the precursor for RhlB to form monorhamnolipids and dirhamnolipids catalyzed by RhlC. The genes (*rhlA*, *rhlB* and *rhlC*) for these catalyses are under the control of QS. Other studies link rhamnolipids synthesis to nutritional conditions, such as nitrogen exhaustion and the alternative sigma factor σ^{54} for nitrogen limitation. Schmidberger et al. [56], reports an interesting study of *P. aeruginosa* and rhamnolipid synthesis. Using PCRq, gene expression was monitored over entire course of fermentation. They observed until late deceleration phase (or ending log phase), an increase in relative gene expression of the *las*, *rhl* and *pqs* quorum-sensing regulon under nitrogen limitation.

Fermentation processes in terms of batch fermentation constituted a molecular black box in regards to transcriptional activity of the genes of LP synthesis circuitry in both Gram-positive and Gram-negative bacteria. More studies of the molecular biology during fermentation of LPs are needed. Monitoring gene expression of LPs over the entire time course of the fermentation process provides information about regulatory events linked or not to QS. QS is a variable that has largely been ignored in fermentative process studies. It is likely that information on QS in fermentation will help optimize bioreactor conditions, nutrient limitations or perhaps the use of signal molecules of QS to improve production yields of LPs and other microbial products.

3. Conclusions

The examples discussed so briefly in this chapter are by no means exhaustive. Hopefully they serve to illustrate the potential use of bacterial LPs and highlight potential applications in fields of biomedicine and agriculture. We also emphasize the potential of endophytic bacteria as lipopeptide-producers, opening research opportunities to understand some of the mechanisms involved in the biological control that occurs in niches they inhabit as endophytes. Knowledge of this and other topics, will promote the implementation of new molecules that are harmless to humans when we cannot directly apply bacteria in agricultural fields. It is clear that for widespread use of microbial LPs, more research is required focused on production with higher yields and at lower cost, where solid-state fermentation emerges as an important area of study in fermentation processes. This field is very much in its early stages, and progress

will come from a combination of ecological, physiological, structural, genetic and fermentative process approaches.

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