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A REVIEW ON THE OPTIMIZATION OF LIPOPEPTIDES PRODUCTION

BY

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Abstract. Lipopeptides are a class of compounds produced by microorganisms, which play a key role in many industries, being used in food, cosmetic and pharmaceutical applications. Their production through biosynthesis is influenced by a large number of factors, including the microorganism characteristics, the operation parameters and the equipment used for separation and purification. The large number of parameters that can affect the biosynthesis makes the process optimization a complex task. This paper aims to present several examples of parameter optimization for the production of lipopeptides through biosynthesis.

Keywords: lipopeptide; biosynthesis; optimization.

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1. Introduction

Lipopeptides are amphiphilic compounds produced by microorganisms that have a large number of possible applications. Their properties and the possible applications have determined a high interest in their industrial production by biosynthesis and on the factors that influence this process.

To this end, numerous studies that aimed to optimize the process for obtaining lipopeptides through biosynthesis have been published recently. A large number of factors that affect the process (characteristics of the microorganism, environmental and technological factors, the equipment used for separation and purification), make the optimization process laborious, requiring the study and specific optimization of each technological process, depending on the microorganism used and on the purity requirements for the final product.

2. Lipopeptides

2.1. The Structure of Lipopeptides

Lipopeptides are biosynthesized by a large number of microorganisms, including bacteria, fungi and actinomycetes. They present an amphiphilic character due to their specific structure: a hydrophobic component and a hydrophilic component and have superficial properties that facilitate their use in a large number of applications.

The structure of lipopeptides consists of a peptide (a chain of amino acids), usually cyclic, of which one or more chains of fatty acids are grafted. Usually, the number of amino acids varies from 7 to 25. This structure underlies the amphiphilic properties of lipopeptides, which can self-assemble into aqueous solutions.

2.2. The Properties of Lipopeptides

Lipopeptides have several interesting features, the most important of which is the amphiphilic character. Lipopeptides partition preferentially between the phases of a liquid, forming a molecular interfacial film lowers the interfacial tension of a solution. From this property other characteristics derive, namely: low critical micellar concentration (the concentration at which the lipopeptide molecules self-assemble into non-covalent aggregates called “micelles”) and the ability to cause a strong decrease in surface tension of the solutions in which they are added (Hamley, 2015).

Along with the surface and self-assembly properties, other properties like dispersion and foaming underlie the use of lipopeptides in various applications, such as surfactants, emulsifying or viscosity reduction agents, controlled–release drug systems etc.

In addition to these properties, their natural origin recommends lipopeptides as non-toxic, biodegradable and even biocompatible substances. Lipopeptides are also resistant to temperature and pH variations (Inès and Dhouha, 2015).

2.3. Possible Applications of Lipopeptides

Lipopeptides are a class of compounds that present a large number of possible applications in various domains, including the food, cosmetic and medical industries. Several examples of possible applications of lipopeptides are presented in Table 1.

Table 1
Possible Applications of Lipopeptides

Lipopeptide family	Lipopeptide	Producing microorganism	Main application
Polymyxins	Polymyxin B	*Bacillus polymyxa*	antibiotics
	Polymyxin E (colistin)	*Bacillus polymyxa*	antibiotics
Streptomyces sp. lipopeptides	Daptomycin	*Streptomyces roseoporous*	antibiotics
Paenibacillus sp. lipopeptides	Fusaricidins	*Paenibacillus sp.*	antibiotics
	Paenibacterin	*Paenibacillusthiaminolyticus*	antibiotics
	Octapeptin	*Paenibacillustianmuensis*	antibiotic
Surfactins	Surfactin	*Bacillus licheniformis* *Bacillus subtilis*	antifungals; antibiotics; surfactants
	Lichenysins	*Bacillus licheniformis*	antibiotics; surfactants
	Pumilacidin	*Bacillus pumilus*	antivirals
Fengycins	Fengicin	*Bacillus subtilis*	biopesticides
	Plipastatin	*Bacillus thuringiensis*	antibiotics
Iturins	Iturin	*Bacillus subtilis*	biopesticides
	Bacillomycin	*Bacillus megaterium*	antifungals
Pseudomonas sp. lipopeptides	Viscosin	*Pseudomonas libanensis* *Pseudomonas fluorescens*	antibiotics; antifungals
	Syringopeptin	*Pseudomonas syringae*	antibiotics
	Xantholysin	*Pseudomonas putida*	antibiotics

Several lipopeptides are used in the treatment of infectious diseases due to their ability to act as Toll-like receptors. These receptors are transmembrane proteins capable of stimulating the immune system response to infectious agents. For example, Pam3Cys lipopeptides were observed in *E.coli* cells,

where they stimulate T lymphocytes to fight against influenza viruses, and Pam3CysSerSer which activate the immune system's response to the virus that causes foot-and-mouth disease. The use of lipopeptides in vaccines is based on the same properties. Currently, vaccines with lipopeptide derivatives are used against HIV, hepatitis B and HPV (human papillomavirus) (Hamley, 2015). Another interesting application of lipopeptides is the synthesis of nanoparticles used as controlled release systems, such as gold or silver nanoparticles (Farias *et al*., 2014) and cadmium sulfide nanoparticles (Singh *et al*., 2011), obtained using surfactin produced by *Bacillus subtilis* and *Bacillus amyloliquifaciens* as a stabilizing agent.

Lipopeptides are also used in the cosmetic industry due to their ability to self-assemble into aqueous solutions. For example, Matrixyl ™ is used in anti-wrinkle products due to its ability to stimulate collagen production in the skin (Hamley, 2015).

Another direction for the use of lipopeptides is to obtain natural pesticides, which, unlike the normal pesticides, have the advantage of being biodegradable and non-toxic. In this regard, three classes of lipopeptides produced by *Bacillus sp.* namely: Surfactin, Iturin, and Fengycin were studied. Surfactin has antibacterial properties, being used as a larvicide, but also in the pharmaceutical and cosmetic industries. Iturin has antifungal properties, being used as a biopesticide, as well as in food and pharmaceutical applications. Fengicin has antifungal activity on filamentous fungi but is inactive against bacteria. In addition to the antibacterial and antifungal effect, these lipopeptides can stimulate nutrient utilization in the soil and induce systemic resistance (Al-Ali *et al*., 2018). In this respect, the introduction of strains of lipopeptide-producing microorganisms into plant crops may represent a solution for the development of organic and sustainable agricultural production (Meena and Kanwar, 2015). They are also applied in oil recovery, crude oil drilling lubricants and bioremediation of water-insoluble pollutants.

The Biosynthesis of Lipopeptides

Lipopeptides are produced by a large number of microorganisms, including bacteria, fungi, and actinomycetes, being mainly involved in the mobility and adhesion capacity of microorganisms on different surfaces.

Among the classes of lipopeptide-producing microorganisms, the most intensively studied are *Bacillus sp.* and *Pseudomonas sp.*, which are used for obtaining most of the lipopeptides with industrial applications. There are also species of actinomycetes known to be capable of synthesizing lipopeptides: *Actinoplanes friulensis* (which biosynthesize friulimicin), *Streptomyces canus* (which biosynthesize amphomycin) and *Streptomyces viridochromogenes* (which biosynthesize laspartomycin).

Due to the fact that lipopeptides are naturally produced by microorganisms, they are suitable for industrial-scale biosynthesis. This consists of the controlled cultivation of a strain of microorganism, under sterile and optimum nutritional and technological conditions, in order to support the main-stream process. The biosynthesis product is then separated from the fermentation liquid by different operations (down-stream process).

Biosynthesis processes present different features depending on the microorganism strain used and on the desired biosynthesis product and are influenced by a large number of factors, including microorganism characteristics, the nutrient and oxygen requirements and technological parameters. Regarding the production of lipopeptides, it can be achieved by liquid fermentation or solid-state fermentation, both methods being intensely studied.

In order to improve the lipopeptide productivity, several techniques were used, including the use of agroindustrial by-products and the optimization of technological parameters. For example, for *Bacillus sp.*, a microorganism that can biosynthesize surfactin, iturin and fengycin, a large number of optimization studies were published. Table 2 presents the maximal concentration obtained for different strains of *Bacillus subtilis*, after optimization.

Table 2
Maximal Concentration Obtained of Bacillus Subtilis Strains after Optimization

Producing microorganism strain	Lipopeptides biosynthesized	Maximal concentration obtained	Reference
B. subtilis BS5	Surfactin	1120mg/L	(Abdel-Mawgoud *et al*., 2008)
Bacillus subtilis BBG131	Surfactin	6527 mg/L	(Motta Dos Santos *et al*., 2016)
Bacillus subtilis SPB1	Iturin and surfactin	85 mg/L	(Ghribi and Ellouze-Chaabouni, 2011)
Bacillus subtilis ATCC 21332	Surfactin	3600 g/L	(Yeh *et al*., 2005)
Bacillus subtilis BBG21	Surfactin and fengycin	2000 mg/L	(Fahim *et al*., 2012)
Bacillus subtilis MI113	Surfactin	350 mg/L	(Ohno *et al*., 1992)
Bacillus subtilis MO-01	Surfactin	1712 mg/L	(Gu *et al*., 2005)
Bacillus subtilis MTCC 2423	Surfactin	1501 mg/L	(Eswari *et al*., 2016)
Bacillus subtilis MZ-7	Surfactin	280 g/L	(Al-Ajlani *et al*., 2007)

4. Optimization of Lipopeptide Production

The main limitation for commercial use of lipopeptides is related to the high production costs and the low yield of product in classical biosynthesis processes. In order to make the industrial use of lipopeptides possible, many efforts have been focused on the optimization of their biosynthesis.

A fundamental feature of biotechnological processes is that the modification of a process parameter determines a chain effect, producing modifications on all the process parameters, which makes it difficult to model and optimize biosynthesis processes.

Any deviation from the optimum parameters has as a direct consequence the modification of the course of the biosynthesis, either by preventing the development of the microorganism, by directing the biosynthesis to obtain another product or by creating difficulties in the separation and purification stage. For this reason, the optimization of biosynthesis processes is intensely studied. The optimization process includes the identification of the microorganism strain with the maximum productivity, the improvement of the biotechnological characteristics of the producing microorganism through genetic engineering techniques, the identification of the optimal technological parameters and the finding of the most suitable separation and purification methods.

4.1. Optimization of Microorganism Development Parameters

Among the parameters that influence the development of the microorganism, the composition of the culture media which ensures the nutrient needs for the growth and development of the microbial mass, is of major interest. Culture media composition includes carbon and energy sources, nitrogen sources, microelements specific for the development of each type of cell, and sometimes, additional nutrients such as vitamins or growth factors.

Between the factors presented, the most effective ones are related to the nutritional needs of the microorganism, out of which the carbon and energy source and the nitrogen source are the major point of interest. In this regard, a large number of studies focused on the improvement of lipopeptide yield by using different components for the culture media. A wide range of carbon and energy and nitrogen sources were tested, including standardized substances or cheaper raw materials like soybean, molasses, cashew apple juice or yeast and meat extract. Several examples of culture media components that were used in optimization experiments are presented in Table 3. The use of a certain source of carbon and energy is imposed both by the ability of the microorganism to metabolize a certain carbohydrate, as well as by the possible inhibitory effect presented by carbohydrates, and by economic reasons. Thus, both standardized substances (glucose, sucrose) and by-products obtained in other industrial

processes (molasses, vegetable residues, by-products obtained from the milk and dairy processing industry, etc.) can be used as carbon and energy sources.

Table 3
Lipopeptide Yield for the Optimum Culture Media Composition

Producing microorganism	Carbon source	Nitrogen source	Lipopeptide yield	Reference
Bacillus subtilis MO-01	Sucrose (22.431 g/L)	Ammonium chloride (2.781 g/L)	1712 mg/L	(Gu *et al*., 2005)
Bacillus subtilis MTCC 2423	Glucose (1.098 g/L)	Yeast extract (0.426 g/L)	1501 mg/L	(Eswari *et al*., 2016)
Bacillus amyloliquefaciens C2LP	Inulin (7.97 g/L)	L-sodium glutamate (16.02 g/L)	99.73 mg/L	(Dang *et al*., 2019)
Bacillus subtilis SPB1	Glucose (40 g/L)	Urea (5 g/L)	720 mg/L	(Ghribi and Ellouze-Chaabouni, 2011)
Bacillus natto NT-6	Glucose (10 g/L)	L-monosodium glutamate (5 g/L)	563.20 mg/L	(Sun *et al*., 2019)
Bacillus subtilis MZ-7	Sucrose (2 g/L)	Yeast extract (3 g/L)	280 mg/L	(Al-Ajlani *et al*., 2007)
Bacillus subtilis BS5	Molasses (160 mg/L)	$NaNO_3$ (5 g/L)	1120 mg/L	(Abdel-Mawgoud *et al*., 2008)
Rhodococcus spp. MTCC 2574	Mannitol (1.6 g/L)	Yeast extract (6.92 g/L) and meat peptone (19.65 g/L)	10900 mg/L	(Mutalik *et al*., 2008)
Bacillus circulans MTCC 8281.	Glucose (32 g/L)	Urea (1 g/L)	4350 mg/L	(Sivapathasekaran *et al*., 2010)
Pseudomonas aeruginosa ATCC 10145	Cashew apple juice (1 g/L)	Peptone (5 g/L)	3860 mg/L	(Rocha *et al*., 2007)
Pseudomonas aeruginosa	Corn steep liquor and molasses (10%)	NH_4NO_3 (2 g/L)	3200 mg/L	(Gudiña *et al*., 2015)
Pseudomonas aeruginosa ATCC-10145	Glucose (1%)	Ammonium nitrate (1 g/L)	1000 mg/L	(El-Sheshtawy and Doheim, 2014)

In general, lipopeptides are obtained on a culture media in which carbohydrates are used as sources of carbon and energy. However, there is the possibility to substitute carbohydrates with hydrocarbons obtained from oil

fractions or with vegetable oils, the lipopeptide-producing microorganisms being able to metabolize these compounds, due to the ability of the lipopeptides to reduce the interfacial tension between the hydrocarbon layer and the aqueous solution of the culture media (Inès and Dhouha, 2015).

An example on the influence of the carbon and energy sources in the culture media on the development of biomass is presented by Al-Ali (2016), who is experimenting the use of a series of complex sugars (glucose, fructose, maltose, xylose, and sucrose), organic acids (succinic acid, fumaric acid, malic acid, oxalic acid, citric acid) and also of glutamic acid on the development of *Bacillus amyloliquefaciens* FZB42 and *Bacillus subtilis* BBG131 strains.

The experimental data showed that the development of the biomass is strongly influenced by the carbon source used. By measuring the optical density of the biomass samples obtained using each of the carbon and energy sources chosen for the two strains, it was concluded that the best sources of carbon and energy for the development of *Bacillus amyloliquefaciens* FZB42 are maltose, sucrose, and xylose, while the use of organic acids results in poorer biomass growth (Al-Ali, 2016).

Regarding the growth and development of the *Bacillus subtilis* BBG131 strain, the best results were obtained with glucose, while the use of organic acids had a negative impact on the growth of the biomass (Al-Ali, 2016).

Another observation made in these experiments is that not only that the development of biomass is influenced by the composition of the culture media, but it also influences the production of lipopeptides. Thus, it was observed that for *Bacillus amyloliquefaciens* FZB42, surfactin production was positively influenced by the use of glucose and fructose as carbon and energy sources, while for *Bacillus subtilis* BBG131, better results were obtained using glucose and sucrose as carbon sources. When using xylose, the lowest concentration of lipopeptide produced was recorded for both bacterial strains (Al-Ali, 2016).

Another culture media component that influences the development of biomass is the nitrogen source. Generally, the culture media must contain an organic nitrogen source which will become a constituent of the bacterial cell. Sometimes, its concentration is supplemented by the addition of inorganic nitrogen sources. It is also important to notice that the ratio between carbon and nitrogen sources (C/N) must be respected since it strongly influences the development of biomass, as suggested by Ghribi and Ellouze-Chaabouni in a 2011 article.

The results of Ghribi and Ellouze-Chaabouni`s experiments showed that, for the strain considered, *Bacillus subtilis* SPB1, between the tested organic nitrogen sources (urea, casein hydrolysate, yeast extract, beef extract, pancreatic casein hydrolysate), the best results were obtained using urea. It was also observed that the C/N ratio strongly influences the growth of biomass and the biosynthesis process, the best results being recorded at a C/N ratio between 6 and 8. At C/N ratios greater than 8, a strong decrease on the surfactant

concentration and in the biomass concentration was observed (Ghribi and Ellouze-Chaabouni, 2011).

The influence of C/N ratio was also studied by Fonseca et al. in a 2007 article. The strain *Bacillus subtilis YRE207* used, isolated from a refinery's petroleum contaminated soil samples, was cultivated in a mineral salts solution, using sucrose as carbon source and NH_4NO_3 as nitrogen source. The influence of C/N ratio was assessed by varying the ammonium nitrate concentration at 4.0, 1.3, and 0.8 g/L, while the sucrose concentration was maintained constant at 10 g/L. The best results were obtained for the C/N ratio corresponding to 3 and 9 (Fonseca *et al*., 2007).

The culture media must also contain mineral salts and metal ions characteristic for the producing microorganism. For example, the synthesis of iturin using *Bacillus amyloliquefaciens* is favored by the presence of Mn^{2+} and Fe^{2+}ions (Lin *et al*., 2008).

It was also observed that other metal cations like Zr^{+4} have little to no effect on the biomass development or lipopeptide concentration (Wei *et al*., 2007). On the other hand, the presence of metallic oxides (CuO, NiO,ZnO) on the culture media has a toxic effect on the biomass while the toxicity of free ions is negligible (Baek and An, 2011).

Also, it is noted that the addition of the substrate can be done progressively, either in stages or continuously, in the latter situation the concentration of the product varying more uniformly, while avoiding the shock produced by the inhibition caused by the addition of a large amount of substrate. From a technological point of view, the optimum variant of adding the limiting substrate is represented by its continuous addition and the maintenance of the concentration between certain limits set (Cașcaval and Oniscu, 2002).

4.2. Optimization of Technological Parameters

One of the most important parameters in the biosynthesis processes is the temperature. Its optimal value varies from species to species and even from strain to strain. In the case of biotechnological processes, there are three temperatures of interest, namely:

- The temperature of biomass growth;
- Respiratory temperature of the cell;
- The biosynthesis temperature.

Because the three phases are difficult to separate, in all the biosynthesis processes, the temperature is chosen as a compromise value between the optimum temperature for biomass development and the temperature at which the microorganism synthesizes the compound of interest, respectively the

isomer of interest (at different temperatures and in the presence/absence of certain components of the culture medium, a microorganism is able to biosynthesize different compounds or isomers of the same compound). Thus, the working temperature can vary between 25°C and 45°C. For example, the optimum temperature for the biosynthesis of surfactin depends on the bacterial strain used: *Bacillus subtilis* (25 - 30°C), *Bacillus coagulans* (28°C), *Bacillus nato* (37°C).

The pH variation can also influence the biosynthesis process, which is why its variation is monitored by the use of pH sensors and controlled by the introduction of pH regulators. In general, the variation of pH within narrow limits acts reversibly on the process. The optimum pH also varies from species to species and from process to process. For example, surfactin biosynthesis using *Bacillus subtilis* is performed at an optimum pH of 6.5-6.8 (Abdel-Mawgoud *et al.*, 2008).

In a 2008 study, Lin *et al*. determined the influence of several factors on the production of iturin using the strain *Bacillus amyloliquefaciens* B128. After optimizing the composition of the culture medium, there were operated changes in the pH value. It was determined that the optimum pH for the successful cultivation of this strain is 6.64, which corresponds to a 3.7% increase in the lipopeptide concentration produced, compared to the reference sample in which no pH changes were performed (Lin *et al*., 2008).

Another important parameter in the management of biotechnological processes with aerobic microorganisms is the amount of oxygen dissolved in the media. Oxygen requirement is a characteristic parameter of each microorganism and the amount of oxygen dissolved in the environment (which becomes accessible to the microorganism for consumption) depends on some factors such as temperature, the morphological structureof the microorganism and the rheological behavior of the fermentation liquid.

In general, the volume of oxygen dissolved in the media strongly influences the aerobic processes, the development of the biomass and the synthesis of the product being positively influenced up to a certain concentration of oxygen, after which it stagnates or decreases. For example, in the case of surfactin production using the *Bacillus subtilis strain* SPB1, testing of several aeration profiles of the fermentation fluid showed that the dissolved oxygen requirement in the environment was around 30%, while the increase of the dissolved oxygen quantity above this value causes the concentration of the biosynthesized lipopeptide to decrease (Ghribi and Ellouze-Chaabouni, 2011).

In order to improve the oxygen intake in the media, the method of air bubbling is used together with mechanical mixing. The rate of dissolution of the oxygen increases with the increase of the stirring, respectively of the flow of bubbled air, but their increase can be made only up to certain limits, determined by the mechanical resistance of the bacterial cells. Also, the biomass growth induces a decrease in the oxygen dissolution rate as a result of

the modification in the rheological behavior of the fermentation liquid (Cașcaval and Oniscu, 2002).

The mechanism of oxygen mass transfer is very complex, involving a large number of stages (the transfer of oxygen from the air bubble into the fermentation fluid, diffusion of oxygen into the fermentation fluid, diffusion of oxygen through the cell membrane), being also dependent on the constructive parameters of the bioreactor.

In regard of the agitation rate, Yeh *et al.* (2005) studied assessed its influence on the surfactin production. The higher agitation rate determined an increase the oxygen mass transfer. However, higher agitation rates determine the formation of foam which decreases the volume of oxygen dissolved in the media. Also, foam formation determines difficulties in the separation and purification stages (Yeh *et al*., 2005).

4.3. Simultaneous Optimization of Multiple Parameters

In regards to the biotechnological processes, there is a strong dependence between the process features, the technological parameters and the development of the microorganism. Often, the variation of one of this factors causing changes in more than one parameter. In this context, the optimization of the biosynthesis processes becomes difficult because, besides the influence of one parameter on the performance criterion pursued (an increase of biomass concentration or the increase in the product concentration), the possible interactions between parameters must be considered. Thus, it is often necessary to find an optimal formula for characterizing a process, which takes into account several parameters, such as the composition of the culture medium, the temperature, the pH or the amount of oxygen dissolved in the media.

An example of multi-parameter optimization is proposed by Jacques *et al*. (1999) which aimed to improve the biosynthesis of lipopeptides using the *Bacillus subtillis* S499 strain. The parameters considered for optimization were the temperature, the pH, the stirring of the fermentation liquid and the concentrations of peptone and yeast extract in a first experiment, respectively the pH, the stirring and the concentrations of phosphate, sucrose, peptone and yeast extract. The obtained results allowed defining the optimal parameters for conducting the biosynthesis process, which are presented in Table 4. The influence of the culture media components was studied by Motta Dos Santos *et al*., in an article from 2016 in which the aim was to improve the biosynthesis of surfactin using *Bacillus subtilis* BBG131 strain. Response surface methodology, a mathematical and statistical method for analyzing the influences of independent variables without knowing the relationship between them, was adopted to optimize the composition of the culture media, using a central composite design with 20 experiments; the input factors considered are the concentrations of glucose, glutamic acid and L-tryptophan.

Table 4
Optimal Parameters for the Biosynthesis of Lipopeptides Using Bacillus Subtilis S499 Strain - after (*Jacques et al., 1999*)

Parameter	Numerical value
Temperature	30°C
pH	7.00
Stirring	200 rpm
Concentration of KH_2PO_4	1.9 g/L
Concentration of microelements solution	1 mL/L
Concentration of sucrose	20 g/L
Concentration of peptone	30 g/L
Concentration of yeast extract	7 g/L

The system behavior and the influence of each factor were expressed by mathematical models generated by specialized software (Design-Expert software). The results obtained led to the conclusion that surfactin production was positively influenced by high concentrations of glucose, glutamic acid, yeast extract, tryptophan and $MgSO_4$, whereas high concentrations of microelements tend to have a negative effect. Data obtained by the Fisher F test suggestedthat the concentration of glutamic acid hadthe greatest impact on surfactin production, results confirmed by other studies (Motta Dos Santos *et al*., 2016).

Another example in this regard is presented by Korayem *et al.* which isolated from the soil a strain of *Streptomyces* producing surfactants. For this strain, the optimization of surfactin production was achieved using the Plackett-Burman method. Testing several sources of carbon and energy (sucrose, glucose, maltose, starch, mannitol, glycerol, and molasses), it was concluded that molasses is the ideal source of carbon. Similarly, the influence on the biomass development of several organic compounds was assessed: organic and inorganic nitrogen sources ($KNO_3, NaNO_3, NH_4Cl, NH_4SO_4$, peptone, yeast extract, and urea), oils (castor oil, cod liver oil, sesame oil, used oil), hydrocarbons (petroleum, diesel, kerosene, toluene, and xylene) and surfactants (EDTA, SDS, Tween 20, Tween 80). It was determined that peptone was the ideal source of nitrogen is while in terms of surfactants, the best results were obtained with Tween 80, oil and residual oil.

The parameters considered further for the optimization were: the concentrations of molasses (g/L), peptone (g/L), residual oil (g/L), oil (%), Tween 80 (%), the temperature (°C), the stirring (rpm), the size of the inoculum (%), the incubation period (days) and the pH. For the 10 parameters considered, the minimum and maximum values were established and 12 experiments were performed with their variations, observing the change of the biosurfactant yield from 29.74% to 42.52% and the positive influence of high concentrations of molasses, peptone and Tween 80 (Korayem *et al*., 2015).

Similarly, Seghal Kiran *et al*. (2010) aimed to optimize lipopeptide production for soil-isolated *Brevibacterium aureum* MSA13 strain. The experiments carried out aimed to identify the best sources of carbon and energy and nitrogen, as well as the concentrations of amino acids and metal ions and optimal values for temperature, pH and the size of the inoculum. In this respect, several sources of carbon and energy represented by industrial by-products (wheat bran, residual sludge from leather processing, residues of seeds from vegetable oils production, treated and untreated molasses) were tested, observing that the useof olives oil and untreated molasses lead to an increase in lipopeptide yield by about 72% (Seghal Kiran *et al*., 2010). As for the nitrogen source, the best results were obtained using acrylamide.

After identifying the most suitable carbon and energy source for the biosynthesis process, the response surface methodology was applied, determining that the most important influence on the process is carried by the quantities of olive oil, acrylamide, ferric chloride added in the culture media and by the size of the inoculums.

Regarding the influence of pH, determined by the introduction of phosphate buffer solution in the fermentation liquid, the optimum was set at pH=7, at the same time observing that the yield in biosurfactant decreased strongly as the pH decreased while increasing the pH above 9 did not determined a strong influence. Also, it was determined that the optimum temperature is 30°C when using molasses as a source of carbon and energy. Comparing the results obtained after the optimization with the results obtained using the original soil isolate strain, productivity improvement of *Brevibacterium aureum* MSA13 was observed to be up to 3x (Seghal Kiran *et al*., 2010).

Nalini *et al.* (2016) proposed a similar methodology for optimizing lipopeptide production using the *Bacillus cereus* SNAU01 strain. Several carbon and energy sources represented by by-products from the vegetable oil industry were tested, determining that the use of coconut oil provided the best results (Nalini *et al*., 2016). Following, to optimize the four parameters of interest (substrate concentration, pH, temperature and size of the inoculum), response surface methodology was applied, using a central composite design with 30 combinations. The experimental data obtained suggested that the mathematical model that describes the behavior of the system is a polynomial equation of the second order. To determine the influence of each parameter, the variation analysis method (ANOVA) was applied. The optimal values identified were: substrate – 8.18 g, inoculum size – 2.5 mL, pH – 7, temperature – 30°C (Nalini *et al*., 2016).

Also, the variation analysis showed that the four parameters have a strong influence on the biosynthesis process. By increasing the volume of the inoculum, an increase in the product yield was observed. Regarding the pH, although the microorganism can biosynthesize the biosurfactant at a pH range

for 5 to 9, the maximum productivity was reached at pH = 7, the variation of pH from the optimum value causing enzymatic changes that can lead to the accumulation of secondary metabolites in the fermentation liquid (Nalini *et al*., 2016).

The existence of a large number of parameters that can influence the process makes the optimization process laborious, requiring a high consumption of resources and time. For this reason, there is a tendency to experimentally test only the hypotheses whose influence is stronger. Thus, using data from the literature and the results of previous experiments, one can predict the behaviour of the system to the variation of some parameters by performing simulations. Such an example is proposed by Alwan *et al*. (2012) which assessed the behaviour of a fermentation liquid at pH, temperature, substrate addition rate (glucose) and dilution rate. Using the Levenberg-Marquardt mathematical model, M. Alwan determined the initial operating parameters, after which changes in these parameters were made. The results obtained allowed the identification of the following aspects:

• the biomass concentration decreases with the increase in the dilution rate, for low and high values of the substrate addition rate;

• the increase of substrate addition rate is followed by the increase of the biomass concentration;

• for low and high values of substrate addition rate, the increase in the temperature determines an increase in the biomass development rate;

• biomass growth is slowed by low values of pH and increases at pH values close to 4 (Alwan, 2012).

These simulations allow the reduction of the number of experiments performed by identifying the parameters that strongly influence the process and also by identifying the corresponding fields of variation. The simulation methods can be successfully coupled with experimental methods, as shown by studies presented in the literature. This approach ensures very good results, with lower consumption of resources and time.

For example, Zheng *et al*. used response surface methodology to optimize the lipopeptide production of *Bacillus subtilis* NEL-01 strain. A five-level three-factor central composite central design was employed to determine the effects of temperature, pH and culture cycle, determining that the maximum lipopeptide yield (1879.56 mg/L) can be achieved at 34.81°C and for a 49.26 h culture cycle (Zheng *et al*., 2013).

Similarly in a 2018 article, Ramya Devi *et al*. used *Pseudomonas guguanensi* in an optimization experiment that aimed to improve the lipopeptide production and the emulsification capacity, using both experimental methods and statistical mathematical models (Response Surface Methodology). Starting from a basic culture medium containing only mineral salts, the authors tested three hypotheses: the use of several carbon and energy sources, organic nitrogen

sources, as well as changes in the pH and duration of the fermentation process. The experimental variants are presented in Table 5.

Table 5
Experiments Performed for the Optimization of Lipopeptide Production by Pseudomonas Guguanensi, after - (*RamyaDevi et al., 2018*)

Basic culture media	Experiment set 1– Changes in the carbon source used	Experiment set 2 – Changes in the nitrogen source	Experiment set 3 – Changes in pH and the fermentation duration	
Nutritive mineral salt solution	olives oil	peptone	pH	Fermentation duration
	vegetal oil	urea	1-14	1-13 days
	coconut oil	peptone and yeast extract		
	n-hexadecane	yeast extract		
	kerosene			
	glucose			

Following the experiments, the best results were obtained using a mixture of peptone and yeast extract as an organic nitrogen source. As sources of carbon and energy, vegetable oil, coconut oil, hexadecane, and glucose were preferred and in terms of pH and duration of the fermentation, the optimum values were set at pH = 8 and 7 days (RamyaDevi *et al*., 2018).

Starting from the data obtained experimentally, an empirical statistical method (Response surface methodology) was applied to determine the possible interactions between these factors, respectively their optimal combination. Based on this, a 24-level central composite design was established for the four factors considered (carbon and energy source, organic nitrogen source, pH and fermentation duration). The interactions between these factors were used to predict maximum emulsification activity and maximum lipopeptide yield, using specialized software (Design Expert Software).

5. Conclusions

Lipopeptides, natural products of microbial metabolism, have numerous industrial applications due to their specific properties. Lipopeptides are of high interest for the pharmaceutical, food, cosmetic, agricultural and even environmental protection industries, but their maximum potential for use is far from being reached.

The progress made in the field of lipopeptides biosynthesis demonstrates that their industrial production is economically and technologically feasible. Experimental design techniques have been extensively used to optimize lipopeptides production. However, there are still many biotechnological processes that require further study and experiments to be improved.

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STUDIUL METODELOR DE OPTIMIZARE A PRODUCȚIEI DE LIPOPEPTIDE

(Rezumat)

Lipopeptidele sunt o clasă de compuși produși de către microorganisme, care joacă un rol important în multe sectoare industriale, fiind utilizate în aplicații alimentare, cosmetice și farmaceutice. Producerea lor prin biosinteză este influențată de un număr mare de factori, incluzând microorganismul, parametrii de operare și echipamentul folosit pentru separare și purificare. Numărul mare de parametri care pot afecta biosinteza fac din procesul de optimizare o sarcină dificilă. Acest articol prezintă câteva exemple de optimizare a parametrilor pentru procese de obținere a lipopeptidelor prin biosinteză.