

Review

Statistical Design, a Powerful Tool for Optimizing Biosurfactant Production: A Review

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Received: 24 July 2018; Accepted: 27 August 2018; Published: 2 September 2018



Abstract: Biosurfactants (Bs) have been studied for decades and applied in different industrial sectors because of their competitive biochemical characteristics, and the fact that they are environmentally friendly. Current scientific investigations mainly involve the search for novel Bs producing organisms with attractive characteristics. Bs are expected to replace synthetic surfactants in the near future, but low production yields and inefficient downstream processes have prevented their widespread use. Although there are numerous reports on Bs optimization, to date there has been no critical compilation or revision of the statistical designs and strategies employed for improved production. The purpose of this mini review is to briefly discuss the factors that affect Bs production and the importance of statistical design as an essential tool for increasing production.

Keywords: biosurfactants; optimization; production; statistical design

1. Introduction

Biosurfactants (Bs) are natural amphipathic compounds produced by microorganisms such as bacteria, yeast, and filamentous fungi [1]. They attract interest for application in diverse industrial areas including the cosmetic, pharmaceutical, agricultural, food, and petrochemical industries, because of their biochemical properties [1–3]. In fact, the global biosurfactant market is expected to reach 2.2 billion US dollars this year, based on a growth rate of 3.5% per annum. Additionally, the global production projection is estimated to reach 476,512.2 tons due to the increasing demand from Asia, Africa, and Latin America, which accounts for 21% of the total production [4].

These secondary metabolites reduce surface and interfacial tension and form micro emulsions solubilizing hydrocarbons in aqueous media, presenting lubricating, wetting, softening, stabilizing, and foaming activities [4,5]. Although they present similar properties to synthetic surfactants they are generally less toxic (with the exception of those presenting antibiotic activity), stable under extreme conditions, they are also biodegradable, and thus, are environmentally friendly [6,7]. Bs are classified into peptides, glycolipids, lipopeptides, fatty acids, phospholipids, and high molecular weight biosurfactants like polymeric biosurfactants [1,8]. Over 200 patents were registered prior to 2011 related with the production of biosurfactants, with 50% of these patents on rhamnolipids, 35% on sophorolipids, and 10% on mannosylerythritol lipids (8).

Even though Bs have shown great potential for widespread use, production and commercialization on an industrial scale has not been fully achieved [5]. Production is difficult because of the high costs

of raw materials, processing costs/purification, and generally low yields. In fact, the raw material costs account for almost 30% of the total production cost [9]. Even so, the number of publications and patents over recent years has considerably increased, but only some have been commercialized [4]. Moreover, presently, the Bs production cost does not compete with those of synthetic surfactants. As a result, different strategies have been proposed, including the development of more cost effective/efficient bioprocesses (optimization production and downstream processing/purification), the use of cheap and waste substrates, and the design of super-producer strains [4,5]. Many publications have focused on increasing production yields in the last two decades [10]. The majority of reports use simple experiments, evaluating one factor at a time (keeping other factors fixed) for improving Bs production, the traditional production strategy [7,11–13]. This conventional strategy is time consuming and may lead to unreliable results and false conclusions. Moreover, carrying out experiments with every possible combination of the variables is not feasible because of the large number of experiments [1,9]. However, there has been an increased tendency to use experimental designs for Bs production optimization in the last decade [5]. The most common statistical designs employed are Response Surface Methodology (RSM) and factorial designs. For optimization, researchers commonly evaluate different nutritional and physicochemical factors that affect Bs production [14]. The nutritional factors generally include the effect of the carbon and nitrogen sources and concentration and their ratios, while the physicochemical factors normally include temperature, shaking, oxygenation, and pH [15,16].

The purpose of this review was to discuss the different statistical approaches for optimizing Bs production used by different research groups. The evaluation of different medium components and physicochemical conditions are also briefly visited.

2. Factors Affecting Bs Production

Before Bs can be produced at an industrial scale, production must be optimized [1]. Thus, the most significant factors that affect Bs production and biochemical properties should be screened for selection and thereupon enhanced. Typically, the nature and concentration of the carbon and nitrogen sources used are the first factors evaluated, followed by choosing an adequate microorganism. However, there are various reports where researchers choose to evaluate other nutritional factors and aspects such as trace elements, and physicochemical parameter such as pH, temperature, and shaking, since they have been shown to have great effect on Bs production and biochemical characteristics in certain cases.

2.1. Strains, Bs Classification and Metabolism

There are many microorganisms that have been reported to produce Bs including fungi, yeast, and bacteria, although bacteria are the most exploited. *Pseudomonas* and *Bacillus* are generally described as super-producers [17]. Other genera such as *Serratia*, *Vibrio*, *Rhodococcus*, *Streptococcus*, *Acinetobacter*, and even *Lactococcus* have also been reported to produce good amounts of Bs [16,18,19]. Additionally, yeast such as *Candida*, *Yarrowia*, *Saccharomyces*, and *Kluveromyces* have been extensively studied for their Bs producing capacities.

The Bs produced by different microorganisms are classified into glycolipids, lipopeptides, fatty acids, phospholipids, polymeric surfactants, and particulate surfactants [17,20]. Each group of Bs can be further divided into sub classifications. For example, glycolipids can be divided into rhamnolipids, trehalolipids, or sophorolipids. Glycolipids are generally produced by a wide range of bacteria and yeast, while other types of surfactants are reduced to a more limited range of bacteria. For example, *Bacillus* is generally prone to synthesize lipopeptide Bs and do not produce glycolipids. *Acinetobacter* and *Pseudomonas* are known to produce a diversity of Bs [17,20].

2.2. Effect of Carbon and Nitrogen Sources

For Bs production, the culture media and culture conditions are extremely important for feasible Bs production. The use of adequate nutrients (carbon and nitrogen sources) directly influences the Bs metabolism, and thus, production [1,7]. Apart from directly participating in cell growth

and reproduction, the carbon and nitrogen sources are used as building blocks for Bs biosynthesis. For example, oily substrates such as coconut oil and soybean oil are used for the biosynthesis of lipidic tails of glycolipids and lipopeptides [21]. While sugars such as rhamnose form part of the polar head of glycolipids. Amino acids have also been found to positively increase Bs production in the case of surfactin and iturin, both of which have polar heads formed of amino acids [22]. Different nutrient sources may regulate Bs synthesis by induction or catabolic repression. Immiscible substrates such as different oils and hydrocarbons have been used as Bs production inducers [23,24]. Catabolic repression has been observed in hydrocarbon degrading bacterial strains that do not produce surface-active molecules in the presence of carbon sources like glucose and organic acids. This can be explained by the fact that hydrocarbon uptake is promoted by Bs production. While sugars such as glucose stimulate biomass growth, and generally Bs are produced in the stationary phase [23]. Although Bs production has been observed to be highest in the stationary phase of microorganisms such as *Bacillus* sp. and *P. aeruginosa* [4], production has also been detected in the exponential phase of bacteria such as *Acinetobacter* sp. (when the bacteria reached the stationary phase, Bs production had already reached its maximum); this has also been reported for *Rhodococcus* sp. and *Aspergillus* sp. strains [10].

Seeing that the carbon and nitrogen sources are vital for Bs biosynthesis, apart from selecting and analyzing the strains of interest, choosing adequate carbons and nitrogen sources is of utmost importance for efficient Bs production. Hence, the importance for the optimization of Bs production using experimental designs. The criteria for choosing adequate carbon and nitrogen sources depends on the producing strain and on the nature of the Bs of interest. In the event of the possibility of using numerous carbon and nitrogen sources, traditional screening or statistical design are implemented. Not only are the significant factors determined, but the levels (concentrations) and relevant interactions are inferred.

2.3. The Effect of Trace Elements on Bs Production

The effect of trace elements (generally metal ions) on Bs production is reported in various articles [25–27]. Phosphates have been shown to affect Bs production. Although little is known about their metabolic interaction, phosphates are important constituents of nucleic acids, phospholipids, and cell walls. Iron has also been shown to affect Bs production since it plays important roles in oxidative phosphorylation [1]. Furthermore, monovalent and bivalent ions such as K^+ , Na^+ , Mg^{2+} , Ca^{2+} , and trace element salts increase Bs yield [4,7].

2.4. Physicochemical Factors Affecting Bs Production: The Effect of pH, Temperature, and Shaking

Bs production is dependent on pH and temperature although Bs production varies with microorganisms. However, optimum Bs production is generally near neutral pH, although some reports suggest a slight increase in pH from 7–8.5 [10,28]. Normally, the best temperatures for Bs production reported are in the range of 35–40 °C [28]. Shaking has also been shown to affect Bs production. The greater oxygenation at higher shaking speed is related to higher metabolism rates [3,29].

3. Statistical Design, an Efficient Tool for Bs Production Optimization

The use of statistical designs have been demonstrated to be a very efficient tool for enhancing biosurfactant production and properties. The most popular designs employed for optimizing Bs production are factorial designs and Response Surface Methodologies (RSM). Factorial designs are multifactor linear models, also denominated “the fully crossed design”. In these designs, the factors evaluated and their levels occur in combination with every level of the other factors. They allow for the measurements of different sorts of factor effect; the main effect (the effect of the independent factors) and the interaction between the factors (how much one factor depends on the level of one or more factors) [30]. The factorial designs reported for Bs production optimization include two-level factorial design, the Plackett–Burman Design (PBD), and the Taguchi design. On the other hand, the RSM is defined as a collection of mathematical and statistical techniques for empirical model building.

By careful design of experiments, the objective is to optimize a response (output variable) which is influenced by several independent variables (input variables). An experiment is a series of tests, called runs, in which changes are made in the input variables in order to identify the reasons for changes in the output response. The application of the RSM design for optimization is aimed at reducing the cost of expensive analysis methods (e.g., the finite element method) and their associated numerical noise [31]. The most common RSM implemented for enhancing Bs production and characteristics are the Central Composite Design (CCD) and the Box–Behnken Design (BBD). Because of the limitation of individual statistical designs, a number of researchers reckon the need for the use of more than one statistical design for best optimization results.

The use of the factorial PBD design is a good option to start with, since it permits the screening and selection of the significant factors on the desired response, in this case Bs production or yield. A second design is then used, for example a RSM like BBD or CCD, to optimize culture conditions and physicochemical parameters. It is important to mention that the results of the strains, factors, and designs used in the literature should not be generalized, since strains, medium component brands, and physicochemical conditions vary, making each study unique. The following sections mention and discuss the statistical approaches used by different authors for Bs production enhancement (see Table 1). The microorganisms and factors evaluated and the effect on production is also briefly analyzed.

Table 1. Summary of different statistical strategies used for optimizing biosurfactants (Bs) production.

Microorganism	Statistical Design	Factor Evaluated	Results	Bs Nature	References
<i>Glycolipids</i>					
<i>Pseudomonas aeruginosa</i>	RSM, 2 ⁴ factorial	Waste free fatty acid waste from soybean oil, NaNO ₃ , PO ₄ ²⁻ , and FeSO ₂ , 7H ₂ O	Maximum yield of 18.7 g dm ⁻¹	Mixture of rhamnolipids	[1]
<i>P. aeruginosa</i>	Single parameter, 2 ⁴ BBD, CCD	Soybean oil, NaNO ₃ , yeast extract, pH, temperature, and shaking	47% increase from 8.6 gL ⁻¹ to 12.6 gL ⁻¹	Possible rhamnolipid	[3]
<i>Klebseilla</i> sp. FKOD36	Taguchi, ANN	Starch, NaNO ₃ , temperature, petrol, pH, incubation period	Yield of 0.038 gL ⁻¹ , EI ₂₄ of 31.67% and ST of 21.6 mNm ⁻¹	Glycolipid and/or phospholipid	[9]
<i>Vibrio</i> sp.	RSM, CCD, AHP	Glucose, sucrose, lactose, maltose, xylose, beef extract, peptone, yeast extraction, soybean meal, corn meal, (NH ₄) ₂ SO ₄ , NH ₄ NO ₃ , NH ₄ Cl, NaNO ₃ , urea	Optimization ST to 41 mNm ⁻¹	Glycoprotien fraction detected	[16]
<i>Rhodococcus erythropolis</i>	ANN and RSM	Sucrose, yeast extract, meat peptone, and toluene	3.5-fold	glycolipid containing trehalose	[24]
<i>P. aeruginosa</i>	PBD, SAD and CCRD	Crude oil, NaCl, (NH ₂) ₂ CO ₃ , MgSO ₄ ·7H ₂ O, NaH ₂ PO ₄ , K ₂ HPO ₄ ·3H ₂ O, EDTA, KH ₂ PO ₄ , (NH ₄) ₂ SO ₄ , C ₃ H ₈ O ₃	ST reduction of 54–27.08 mNm ⁻¹	Glycolipid, rhamnolipid	[32]
<i>Psuedomonas</i> sp.	PBD	Carbon source, nitrogen source, C/N ratio, iron concentration, magnesium concentration, phenol toxicity, pH, temperature, shaking, sampling time	2-5 fold increase	Glycolipid, rhamnolipid	[33]
<i>Pseudomonas putida</i>	BBD	Glucose, ammonium chloride, yeast extract	50 mgL ⁻¹	Glycolipid, rhamnolipid	[34]
<i>P. aeruginosa</i>	PBD and BBD	Sawdust, glycerol, groundnut husk, groundnut oil, pH, inoculum size	Reduction in surface tension from 68.72–39.11 mNm ⁻¹	Glycolipid, rhamnolipid	[35]
<i>P. aeruginosa</i>	PBD, SAD and BBD	Glycerol, methanol, ethanol, mannitol, glucose, sucrose, starch, soybean oil, sunflower oil, NH ₄ Cl, NH ₄ NO ₃ , (NH ₄) ₂ SO ₄ , urea, NaNO ₃	3089 mgL ⁻¹	Glycolipid, rhamnolipid	[36]

Table 1. Cont.

Microorganism	Statistical Design	Factor Evaluated	Results	Bs Nature	References
<i>Lipopeptides</i>					
<i>Bacillus</i> sp.	Modified Gompertz Model	Glucose, ammonium sulphate	Accurate production prediction	Cyclic lipopeptide, Surfactin	[15]
<i>Bacillus licheniformis</i>	PBD and BBD	Glucose, CaCl ₂ , H ₃ PO ₄ , H ₃ BO ₃ , CuSO ₄ , ZnSO ₄ , CoCl ₂ , Na-EDTA, NaNO ₃ , MgSO ₄ ·7H ₂ O, KCl, MnSO ₄ , and Na ₂ MoO ₄	10-fold increase in Bs production and Critical micelle dilution	Cyclic lipopeptide, Surfactin	[25]
<i>Bacillus</i> sp.	PBD	Frying oil waste, sucrose FeSO ₄ ·7H ₂ O, NaNO ₃ , KH ₂ PO ₄ , K ₂ HPO ₄ , MgSO ₄ ·7H ₂ O, ZnSO ₄ ·7H ₂ O, MnSO ₄ ·4H ₂ O, NH ₄ NO ₃ , and CaCl ₂ ·2H ₂ O	124% increase in production	Cyclic lipopeptide, Surfactin	[26]
<i>Bacillus subtilis</i>	Taguchi	K ⁺ , Mg ²⁺ , Ca ²⁺ , Fe ²⁺ , Mn ²⁺ , Na ⁺	3.34 gL ^{−1}	Cyclic lipopeptide, Surfactin	[27]
<i>Bacillus mycoides</i>	CCD	Temperature, pH, salinity, and glucose	ST reduction from 61 to 34 mNm ^{−1}	lipopeptide	[28]
<i>B. subtilis</i>	2 ³ factorial design	Sucrose, NaNO ₃ , (NH ₄) ₂ SO ₄ , (NH ₄) ₂ NO ₃ , urea, residual brewery yeast	ST reduced to 29.3 mNm ^{−1}	Cyclic lipopeptide, Surfactin	[37]
<i>B. subtilis</i>	2 ³ factorial design CCD	Olive leaf residue flour, olive cake flour, inoculum size, and moisture content	30.67 mgg ^{−1}	Cyclic lipopeptide, Surfactin	[38]
<i>B. subtilis</i>	BBD	Primary inoculum age, secondary seed culture age, and size	3.4 gL ^{−1}	Cyclic lipopeptide, Surfactin	[39]
<i>Brevibacterium areum</i> MSA13	RSM	Olive oil, ferric chloride, inoculum size, acrylamide	3-fold	Lipopeptide, Brevifactin	[40]
<i>Partially identified</i>					
<i>Serratia marcescens</i>	BBD 3 ³ factorial	C/N, C/Fe, and C/Mg ratio	ST reduction from 66–31 mNm ^{−1} and a yield of 4.1 gL ^{−1}	Unidentified, Possible lipopeptide	[18]
<i>Lactococcus lactis</i> and <i>Streptococcus thermophilus</i>	Fractional Factorial Design, SAD and CCD	Peptone, meat extract, yeast extract, lactose, ammonium citrate and KH ₂ PO ₄ , Lactose, soya peptone, and sodium glycerophosphate	1.8 and 2.1-fold increase for <i>Lactococcus lactis</i> and <i>Streptococcus thermophilus</i> , respectively	Unidentified, possible sophorolipids	[41]
<i>Rhodococcus</i> spp. MTCC 2574	The one-at-a-time approach and CCRD	Mannitol, yeast extract, and meat peptone	3.2 to 10.9 gL ^{−1} ST tension of 72 to 30 mNm ^{−1} .	unidentified, protein and carbohydrate fraction	[19]
<i>Streptomyces</i> sp.	CCRD	pH and temperature	Production yield of 1.74 gL ^{−1} and a ST of 25.34 mNm ^{−1}	Unidentified glycoproteic fraction	[42]

Table 1. Cont.

Microorganism	Statistical Design	Factor Evaluated	Results	Bs Nature	References
<i>Unidentified</i>					
<i>Acinetobacter</i> sp. YC-X 2	One-factor RSM, CCD	Beef extract, peptone, NaCl and n-hexadecane	57.5% increase	Unidentified	[10]
<i>Yarrowia lipolytica</i>	2 ⁴ factorial, RSM	Urea, ammonium sulfate, yeast extract, peptone, glycerol, hexadecane, olive oil, and glucose	110.7% increase in EI ₂₄ and 108.1% decrease in ST	Unidentified	[14]
<i>Candida lipolytica</i>	2 ³ factorial design (RSM/CC)	Agitation, aeration, and time of the process	0.59–7.27 gL ^{−1} yield	Unidentified	[29]
<i>Streptomyces</i>	PBD	Starch nitrate medium, molasses, peptone, Tween 80, incubation period, inoculum size	13.5% increase in EI ₂₄ , from 31.74–42.68%	Unidentified	[43]
<i>Bacillus brevis</i>	RSM CCD	Temperature, pH, incubation period, and glucose concentration	Emulsion of 28.8–73%	Unidentified	[44]
<i>Bacillus circulans</i> MTCC 8281	ANN	Glucose, Urea, SrCl ₂ , and MgSO ₄	70% increase to 4.38 gL ^{−1}	Unidentified	[45]
<i>Lactobacillus pentosus</i>	BBD	pH, temperature, and salinity	ST reduced from 69.3–53.8 nM/m. Emulsion volume of 45.93% and emulsion stability of 100%.	Possible glycolipopeptide	[46]
<i>Ochrobactrum intermedium</i>	PBD and BBD	pH, temperature, molasses, MgSO ₄ , Waste engine oil, Waste cooking oil, K ₂ HPO ₄ , Olive oil, CaCl ₂ , Whey, Yeast extract	1.89-fold increase in EI ₂₄	Unidentified	[47]
<i>Lactobacillus pentosus</i>	BBD	Operation time, temperature, and salt concentration	Bs yield improved from 9.49–13.76 mgL ^{−1}	Possible glycolipopeptide	[48]

* Abbreviations: Analytical Hierarchy Process (AHP); Artificial Neural Network (ANN); Box-Behnken Design (BBD); Contour Curve (CC) Central Composite Design (CCD) or Central Composite Rotatable Design (CCRD); Plackett-Burman Design (PBD); Response Surface Methodology (RSM); Steepest Ascent Design (SAD).

4. Two-Level Factorial Designs

These are designs for 2–21 factors where each factor is varied over two levels. They are useful for estimating the main effects and interactions (Design Expert 7, Stat-Ease, Inc., Minneapolis, MN, USA). Generally, factorial designs are used along with RSM.

4.1. Plackett–Burman

This is a specialized design for 2–31 factors where each factor is varied over two levels. It can be used if the absence of two-factor interactions can be assumed, otherwise a higher resolution factorial design is used. The PBD design is useful for testing ruggedness where little or no effect on the response due to the factors is expected. Thus, the first step needed to optimize the production of microbial metabolites can be done by screening the factors that affect the response of interest. The Plackett–Burman factorial design permits screening of numerous factors (without the interactions), including nutrients and physicochemical parameters, and thus, reduces the number of factors to be considered and analyzed in the subsequent optimization experiments [25,26,32]. The PBD is a good option for starting optimization of Bs production since the number of factors influencing production are multifactorial, and optimal production depends not only on the source and concentration of nutrients for example, but also on the bacterial specie and strain studied.

In a study carried out by Praharyawan et al. [26], a PBD was used to optimize Bs production by *Bacillus* sp. Of the factors analyzed (frying oil waste, sucrose $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, NaNO_3 , KH_2PO_4 , K_2HPO_4 , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, NH_4NO_3 , and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$), all factors were significant except for $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ and NH_4NO_3 . Their analysis revealed that $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ had the greatest significance. They argued that the Zn^{2+} and Mg^{2+} negatively affected bacterial growth, and since bacterial growth was directly proportional to Bs production, high concentrations negatively affected Bs production. On the other hand, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ positively affected Bs production, an observation that was in accordance to other reports on surfactin by other *Bacillus* strains. $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ showed a positive effect on Bs production and might have been related to its effect on the cell membrane. NaNO_3 and the frying oil waste were also essential for Bs production, and this was supported by the fact that there is a lot of evidence that suggests that these components are used as substrates for biosynthesis. Other interesting findings were the fact that KH_2PO_4 had a negative effect on Bs production while K_2HPO_4 favored production. The optimization of this culture medium resulted in a significant increase in Bs production by 124% compared to the standard non-optimized medium.

Hassan et al. [33], carried out PBD to optimize rhamnolipid production by two *Pseudomonas* sp. strains. The effects studied were carbon source, nitrogen source, C/N ratio, Fe^{2+} concentration, Mg^{2+} concentration, phenol toxicity, pH, temperature, shaking, and sampling time. The first design (evaluating carbon sources) allowed for an increase in Bs production in the range of 2–5 fold (depending on the strain), while the second design (carbon source concentration) showed no significant increase. For rhamnolipid synthesis, coconut oil had the greatest effect, while there was no discrimination in the nitrogen source for Bs synthesis. Low magnesium concentration favored Bs production suggesting that protein synthesis was blocked and cellular metabolism was shifted to carbohydrate synthesis and rhamnolipid production. This statistical design also revealed that for rhamnolipid production of these *Pseudomonas* strains, the physicochemical properties had a significant effect. Relatively low temperatures (30 °C), slight alkaline pH (8), and high agitation favored Bs production.

In another study, a PBD was used to optimize Bs production of *Streptomyces* sp. isolated from Egyptian arid soil. The most significant factors evaluated were starch nitrate medium, molasses, peptone, Tween 80, incubation period, and inoculum size, increasing emulsion by 13.5% [43].

4.2. Other Two-Level Factorial Designs

These are designs used for 2–21 factors where each factor is varied over two levels. They are useful for estimating the main effects and interactions (Design Expert 7, Stat-Ease, Inc., Minneapolis, MN, USA).

A 2^3 factorial design was implemented to improve Bs activity of a surfactin produced by *Bacillus subtilis*. Sucrose was evaluated as the carbon source and NaNO_3 , $(\text{NH}_4)_2\text{SO}_4$, NH_4NO_3 , urea, and residual brewery yeast were used as the nitrogen sources. NH_4NO_3 was the most important nitrogen source for Bs production. The C/N ratio also had an impact on Bs production. Surface tension (ST) was reduced to 29.3 mNm^{-1} [37].

In order to optimize Bs production in *B. subtilis* SPB1 under solid-state fermentation, making production cost effective, a 2^3 factorial design and a central composite were applied [38]. To enhance Bs production, olive leaf residue flour, olive cake flour, inoculum size, and moisture content were analyzed. Bs production reached 30.67 mgg^{-1} .

Statistical designs have also been used for optimizing Bs production of yeast. Fontes et al. [14] used a 2^4 complete factorial design and RSM to optimize *Yarrowia lipolytica* IMUFRJ 50,682 Bs production. The effect of nitrogen sources (urea, $(\text{NH}_4)_2\text{SO}_4$, yeast extract, and peptone) were evaluated first, and then the carbon sources (glycerol, hexadecane, olive oil, and glucose). In terms of the nitrogen sources used, yeast extract and $(\text{NH}_4)_2\text{SO}_4$ favored Bs production at low levels. The authors argued that this was probably due to the fact that when nitrogen was in excess, Bs production decreased, since carbon was used for cell growth. Glucose and glycerol were the most significant carbon sources (at intermediate levels), while olive oil and hexadecane did not favor Bs production. An increase in shaking resulted in an increase in Bs production, and the authors suggested that this was due to an increase in oxygenation levels. Optimization by statistical design enhanced ΔEI_{24} by 110.7% and ΔST by 108.1% in relation to the standard process.

A 2^3 factorial design (RSM with Contour Curve) was also used for optimizing Bs production in the yeast *Candida lipolytica* UCP0988. Physicochemical parameters were evaluated. The input variables were agitation, aeration, and time of the process, and the output variables were the ST, yield, and biomass. The results of the analysis revealed that although time had a positive effect on Bs production yield, agitation and aeration had a negative effect with an optimum yield of 7.27 gL^{-1} [29].

Trace element composition was optimized by the Taguchi method for improved surfactin production in *B. subtilis* [27]. This is an orthogonal array design from Taguchi's textbook which explores two level factorial and general factorial designs as alternatives. Mg^{2+} , K^+ , Mn^{2+} , and Fe^{2+} were found to have the greatest effect on Bs production, since the absence of these ions in the culture medium resulted in a decrease in production yield of $0.4\text{--}0.6 \text{ gL}^{-1}$. The Taguchi approach increased production yields from 1.74 gL^{-1} of the control experiments to 3.34 gL^{-1} in the optimized medium.

5. Response Surface Methodology (RSM)

The RSM includes factorial designs and regression analysis, and efficiently deals with multifactor experiments. RSM involves a number of statistical techniques for designing experiments, building models, and evaluating the effects of the factors of interest [1].

5.1. Central Composite Design

RSM that are most commonly used for enhancing Bs production include the CCD and the BBD, although the one-factor strategy has been used. For the CCD each numeric factor is varied over five levels: plus and minus alpha (axial points), plus and minus 1 (factorial points), and the center points. In the BBD design each numeric factor is varied over three levels.

Chen et al. [10] decided to optimize the production of a Bs from *Acinetobacter* sp. YC-X 2 that showed stability under high temperatures, salinity, and harsh pH conditions. In order to determine the factors that had a significant effect on Bs production a one-factor RSM was used. Once optimal

conditions were obtained, a CCD quadratic response model obtained with experimental data was implemented. Analysis of the data showed a 57.5% increase in Bs production with beef extract 3.12 gL^{-1} , peptone 20.87 gL^{-1} , NaCl 1.04 gL^{-1} , and *n*-hexadecane 1.86 gL^{-1} . According to the model, peptone was the most significant factor. Bs production was directly related to cell growth where the highest emulsion values were at high concentrations of peptone and beef extract. Bs production was stimulated at lower levels of hexadecane and NaCl.

A RSM (CCD) optimized Bs production by *B. brevis*. The effect of temperature, pH, incubation period, and glucose concentration were evaluated [44]. Emulsion reached 73%. All the factors studied had an influence of Bs production. Long incubation periods, slightly alkaline pH (8) (interaction incubation-period-pH), and intermediate temperatures ($33 \text{ }^{\circ}\text{C}$) (interaction incubation period-temperature) favored Bs production. High glucose concentrations and slightly alkaline pH influenced Bs production (interaction glucose-pH). The interaction between glucose concentration and incubation periods was not very significant.

The production of a Bs of a glycoproteic nature was enhanced using the CCRD method [42]. The *Streptomyces* sp. DUPA1559 strain was isolated from lichens in the Amazon region in Brazil. These Bs showed promising biochemical properties like low Critical Micelle Concentration (CMC), high thermal and pH stability, salt tolerance, and low toxicity, thus production was optimized. Temperature and pH were the variables analyzed using 12 experiments with four replicates at the central points. Statistical analysis showed that pH had a greater effect than temperature on ST. Under optimized conditions the Bs reduced ST to 25.34 mNm^{-1} compared to 27.14 mNm^{-1} . The Bs production yield reported was 1.74 gL^{-1} after 96 h.

Najafi et al. [28] also applied a CCD to enhance the Bs production (lipopeptide) from an indigenous strain of *Bacillus mycoides*. Temperature, pH, salinity, and glucose were the factors evaluated. Although this strain produced Bs over a wide range of temperatures, maximum Bs production was at a higher temperature ($39.03 \text{ }^{\circ}\text{C}$), suggesting that the bacteria could be a moderate thermophile. Their analysis showed that low salt concentrations negatively affected Bs production. Glucose also positively affected Bs production. ST was reduced from 61 to 34 mNm^{-1} .

5.2. Box–Behnken

In this design each numeric factor is varied over three levels. If categorical factors are added, the Box–Behnken design will be duplicated for every combination of the categorical factor levels. These designs have fewer runs than three-level factorials (Design Expert 7, Stat-Ease, Inc., Minneapolis, MN, USA).

A Box–Behnken 3^3 factorial design was used to optimize Bs production in *Serratia marcescens*. The C/N, C/ Fe^{2+} , and C/ Mg^{2+} ratios at three different levels were evaluated. For the interaction between C/ Fe^{2+} and C/N, the greatest reduction in ST was when both ratios were highest. In terms of the interaction between C/ Fe^{2+} and C/ mg^{2+} , the same pattern was observed. With the use of this design, ST was reduced to 31 mNm^{-1} with a yield of 4.1 gL^{-1} after 48 h [18]. Martínez-Toledo and Rodríguez Vázquez [34] employed a BBD evaluating the effect of glucose, NH_4Cl and yeast extract on phenanthrene removal by *Pseudomonas putida* CB-100. Rhamnolipid production was also measured, and their results showed that high concentrations of glucose and NH_4Cl favored Bs synthesis, reaching 50 mgL^{-1} after 10 h.

A *B. subtilis* strain lipopeptide production was optimized to 3.4 gL^{-1} using a BBD [39]. Primary inoculum age, secondary seed culture age, and size were the factors evaluated. These authors emphasized that although most studies for improvement of Bs production focuses on nutritional factors the physical parameters also have a great importance for increasing the cost-effectiveness of biotechnological processes. Their analysis showed that all the factors and the interaction between the factors were significant, and that increasing the secondary seed culture negatively affected Bs production. A lower initial cell density also increased Bs productivity. This was explained by the fact that in the case of sporulating bacteria like *B. subtilis*, less and younger biomass would lengthen the

exponential phase where the Bs can be produced; since once the stationary phase is reached sporulation processes are induced [39].

Brevifactin (lipopeptide) production by *Brevibacterium aureum* MSA13 in a solid-state culture was optimized by RSM. After a number of factors affecting Bs production were initially traditionally screened (one-at-a-time), olive oil, FeCl_3 , inoculum size, and acrylamide were selected for optimization. Different industrial wastes were also chosen for the base formulation of the media culture including oil seed cake, wheat bran, tannery treated sludge, tannery pre-treated sludge, treated molasses, and pre-treated molasses. The ANOVA analysis after optimization showed that inoculum size, and acrylamide (nitrogen source) were the most significant factors, followed by olive oil and FeCl_3 [40].

Vecino Bello et al. [46] obtained data that enabled them to develop a second-order model describing the interrelationship between operational and experimental variables by the use of different equations (linear, interaction, and quadratic terms). They implemented Box–Behnken factorial design to evaluate the effects of pH, temperature, and salinity on Bs produced by *Lactobacillus pentosus*. The pH had the most prominent effect on ST reduction and emulsion stability. However, their analysis showed a synergistic effect in reducing ST between pH (3–5.5) and low salt concentrations and low temperature. Interestingly the opposite synergistic effect was observed at pH 8. After optimization ST was reduced to 53.8 from 69.3 nM/m. Emulsion volume reached 45.93% and emulsion stability was optimized to 100%.

6. Modified Gompertz Equation

The Gompertz model is well known and widely used in many aspects of biology. It has been frequently used to describe the growth of animals and plants, as well as the number or volume of bacteria and cancer cells [49]. However, an interesting study with a very different and novel approach was carried out by Heryani and Putra [15]. For the first time, a modified version of the Gompertz model was used to predict the cell mass and Bs production by *Bacillus* sp. strains. This model was also demonstrated to precisely predict ST. The results showed that glucose had a negative effect on surfactin production (high concentrations of glucose inhibited Bs production). The optimum C/N ratio was also determined using this method. Maximum Bs production reached 2.46 gL^{-1} with the lowest ST recorded at 27.05 mNm^{-1} .

7. Mixed Strategies

Most researchers who have carried out studies on Bs production optimization or enhancement of Bs biochemical characteristics have considered the limitation of using only one statistical approach. The following examples review the use of more than one statistical design, generally involving prior screening of significant factors followed by medium optimization and improved Bs production yields.

Abalos et al. [1] optimized a mixture of rhamnolipids produced by *Pseudomonas aeruginosa*, using a 2^4 full factorial design and CCD for the response surface modeling method (RSM). These authors determined that the carbon source (refined soybean oil), nitrogen source (NaNO_3), potassium phosphate salts (mono and dibasic), and FeSO_4 were the factors that significantly affected Bs production. Under optimum culture conditions, Bs production reached 18.7 gdm^{-1} . They concluded that at high concentrations of the medium components, biomass was favored and that Bs production was favored when medium components were low (except for the carbon source).

Another very interesting study was carried out by Abbasi et al. [3]. Their strategy for finding the optimum conditions for maximum Bs production by *P. aeruginosa* was complete. Since the factors affecting Bs production were unknown, they carried out preliminary experiments to determine the factors that may have had an effect on Bs production. First, they evaluated the carbon, nitrogen, and physicochemical parameters based on single parameter experiments (traditional approach). Overall, 19 carbon sources and 11 nitrogen sources were screened. Once the most effective carbon and nitrogen sources were determined, Bs production was optimized in two stages using RSM approaches. First, the authors carried out a 2^4 factorial design and BBD, with the factors selected from

the preliminary data. Afterwards, the CCD was implemented, based on the key medium components for Bs production; previously determined in the BBD. Analysis of this study showed that soybean oil and NaNO_3 were the most effective carbon and nitrogen sources. However, yeast extract (used as a complementary nitrogen source) was vital for maximum Bs production. Among the physicochemical factors, only temperature and shaking had a significant effect on Bs production; pH and inoculum size had no significant effect. RSM optimization of medium components resulted in an increase in Bs by 47% yielding 12.6 gL^{-1} , as compared to the best yield using the single factor approach (8.6 gL^{-1}). This Bs reduced the ST to 32.5 mNm^{-1} and presented a CMC of 10.1 mgL^{-1} .

Tian et al. [32] carried out a simple but very effective study optimizing *P. aeruginosa* Bs production and reducing production costs. They employed a PBD, followed by a Steepest Ascent Design (SAD) and a CCD. Out of the 10 factors analyzed, only NaCl, $(\text{NH}_4)_2\text{SO}_4$, and crude oil were significant, and thus, they were used for further optimization. At the end of the study, ST was reduced to 27.08 mNm^{-1} .

Kumar et al. [35] also applied a PBD to determine the factors that had an effect on Bs production by *P. aeruginosa* 2297. Sawdust, glycerol, and pH were defined as the important factors and their concentrations were optimized using a 2^3 factorial BBD (RSM) to optimize Bs production. Groundnut husk, groundnut oil, and inoculum size had no effect on Bs production. In this particular study ST was reduced to 39.11 mNm^{-1} .

In the same way, Deepika et al. [50] used different statistical designs for optimization of rhamnolipid production by *P. aeruginosa* KVD-HR42. This Bs was of interest since like other rhamnolipids it showed high stability under extreme conditions of temperature, pH, and NaCl, as well as an attractive CMC. As in other cases, a PDD design was used to determine the significant factors (Karanja oil, NaNO_3 and pH). Afterwards, RSM was performed using a BBD. After optimization, Bs production yield was 5.9 gL^{-1} after only 48 h, at 37°C .

Rhamnolipid production by *P. aeruginosa* J16 was optimized by a mixture of experimental designs [36]. First, different nutrients were screened to determine which ones had significant effects on Bs production. Afterwards, a SAD was used, and finally the RSM. From a total of 9 carbon sources (glycerol, methanol, ethanol, mannitol, glucose, sucrose, starch, soybean oil, and sunflower oil) glycerol and glucose had the most significant effect, followed by soybean oil and sunflower oil. Bs yield oscillated between 506 and 678 mgL^{-1} . *P. aeruginosa* was not able to grow on methanol, sucrose, and starch. Then, five nitrogen sources (NH_4Cl , NH_4NO_3 , $(\text{NH}_4)_2\text{SO}_4$, urea, NaNO_3) with glucose and glycerol were evaluated by the one-factor-at-a-time. This design uses one numerical factor and three levels for a lineal model, five levels for a quadratic model, seven levels for a cubic model, plus some replicated points (Design Expert 7, Stat-Ease, Inc., Minneapolis, MN, USA). The highest yield (2121 mgL^{-1}) was obtained with glycerol and $(\text{NH}_4)_2\text{SO}_4$. Glycerol, $(\text{NH}_4)_2\text{SO}_4$ and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ were selected based on the previous PBD for optimization by SAD and RSM (BBD) producing 3037 and 3089 mgL^{-1} , respectively. These values were obtained in intermediate levels of the three components used.

In another study, Bs production by *Bacillus licheniformis* K51 was optimized by initially screening 14 factors components [25]. CaCl_2 , H_3PO_4 , H_3BO_3 , CuSO_4 , ZnSO_4 , CoCl_2 , and Na-EDTA were found to be significant, while glucose, NaNO_3 , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, KCl, MnSO_4 , and Na_2MoO_4 were not. Subsequently, a BBD was implemented to optimize Bs production. The most significant factors with a positive effect (CoCl_2 and H_3PO_4) and with a negative effect (H_3BO_3 and Na-EDTA) were used for the design. The RSM showed that for the interaction between CaCl_2 and H_3PO_4 , Bs yield was higher at higher concentrations of these components, while for the interaction between H_3BO_3 and Na-EDTA the effect was the opposite. Bs production was increased 10-fold.

These two strategies (PBD and followed by a BBD) were implemented to screen and optimize the most important factors lipase and Bs production in *Ochrobactrum intermedium* [47]. Like with the other examples, the factors that had the greatest effect on production (determined by the PBD) were used for optimization. In this particular study, out of 11 factors (both nutritional and physical), only pH, temperature, and molasses were the significant factors. These authors improved Bs production

1.89-fold. Production time was also shorter, making production more feasible. The nature of the Bs was not mentioned.

A mixture of statistical designs have been used for increasing the production of Bs (Sphorolipids) in proteobacteria like *Lactococcus lactis* and *Streptococcus thermophilus* [41]. The designs used were Fractional Factorial Design (FFD) (2^{6-2}), SAD, and CCD. Six major components of the MRS (DeMan, Rogosa and Sharpe) medium (peptone, meat extract, yeast extract, lactose, ammonium citrate, and KH_2PO_4) and M17 medium (peptone, meat extract, yeast extract, lactose, soya peptone, and sodium glycerophosphate) were evaluated. Bacterial growth was directly related to Bs production, thus, the optimum conditions for biomass production were used for Bs production. Lactose and peptone were the factors that mostly influenced cell growth. Bs production was maybe stimulated under limiting conditions in the stationary phase. After optimization, Bs production increased 1.8 and 2.1-fold for *Lactococcus lactis* and *Streptococcus thermophilus*, respectively.

Mutalik et al. [19] enhanced Bs production of *Rhodococcus* spp. MTCC 2574 by combining traditional and statistical methodologies. Firstly, seven *Rhodococcus* strains were screened for Bs production (using the one-at-a-time approach) and one was selected. Mannitol, yeast extract, and meat peptone were used as the carbon and nitrogen sources. Additionally, *n*-hexadecane was used as an inducer. Afterwards, the most important factors were selected for optimization using a CCRD. Bs production yield increased 3.4-fold with initial and final values of 3.2 and 10.9 gL^{-1} , respectively. ST tension was reduced from 72 to 30 mNm^{-1} . The authors argued that mannitol could have acted as a primary source for initial bacterial growth, while hexadecane may have been used in the latter stages of growth.

Recently, Rosas-Galván et al. [51] studied the production Bs by *Serratia marcescens* SM3 and its isogenic SMRG5 strain. The effect of the carbon and nitrogen sources on the production of two different lipopeptides (octadecanoic and hexadecanoic acid for SM3 and SMRG5, respectively) were evaluated. Glycerol was the carbon source studied while $(\text{NH}_4)_2\text{SO}_4$ and peptone casein were the nitrogen sources evaluated. The statistical approach used was a 2^3 factorial design followed by the RSM. An interesting observation from this study was that the same factors evaluated had varying effects on the two *S. marcescens* strains. Bs production in SM3 (represented by a reduction in ST and increase in EI_{24}) was favored at high concentrations of glycerol and peptone, while the opposite was reported for the isogenic strain. After the application of the statistical designs, ST was reduced to 26.5 and 25.2 mNm^{-1} , for SM3 and SMRG5, respectively. Likewise, the EI_{24} reported were 79.9 and 89.7%, for SM3 and SMRG5.

7.1. Analytical Hierarchy Process (AHP)

The AHP is a technique for organizing the information and judgment used in making complex decisions. The AHP can solve problems especially where multiple factors and choices (or alternatives) have to be considered simultaneously. In a study carried out by Hu et al. [16], RSM and AHP were used to determine the optimum medium composition for Bs production by *Vibrio* sp. 3B-2. Before optimization, one-factor experiments were carried out (evaluating temperature, culture size, and inoculum size) to find the best conditions for further optimization. Afterwards, different carbon sources (sugars) and nitrogen sources (complex organic and inorganic) were evaluated. After optimization using carbon and nitrogen sources, different salts (ZnSO_4 , Na_2HPO_4 , CaCl_2 , BaCl_2 , CuSO_4 , MgSO_4 , MnSO_4 , and FeCl_3) were evaluated based on one factor experiments. The authors decided to use the AHP approach in order to evaluate the importance of each factor. Overall, maltose, xylose, and lactose were the carbon sources that favored most Bs production. Yeast extract was the nitrogen source that mostly favored Bs production. Among the salts, NaCl had the greatest effect of Bs production. According to the authors, NaCl inhibited bacterial growth, and thus, favored Bs production. This phenomenon has been observed in other studies whereby Bs production is inversely proportional to growth. Although Na_2HPO_4 was the best trace element for Bs production, the other trace element ions were also necessary for Bs production, since the lack of these elements disrupted Bs production.

7.2. Artificial Neural Network

Although, many statistical designs such as PBD, RSM using CCD, and factorial designs including the Taguchi design are usually adequate for Bs optimization, in some cases, complex non-linear biological interactions cannot be fully exploited. In this case, the use of advanced computational methods based on artificial intelligence can be used to predict Bs production using their dependent variables. Such is the case of a report by Ahmad et al. [9], who used the Artificial Neural Network (ANN) which is used for simulating complex system performance based on limited experimental data. ANN has been shown to be a powerful tool for optimizing Bs production. In this particular study, the authors estimated Bs yield, ST reduction, and emulsion by *Klebsiella* sp. FKOD36. Along with the ANN, a Taguchi design was used and temperature, pH, incubation period, nitrogen, carbon, and hydrocarbon sources were used as variables. The most efficient ANN model showed a yield of 0.038 gL^{-1} , with and emulsion index and ST reduction of 31.67% and 21.6 mNm^{-1} , respectively. Sivapathasekaran et al. [45] also used the ANN strategy with *Bacillus circulans* MTCC 8281. After determining the most important factors for Bs production (glucose, urea, SrCl_2 , and MgSO_4), ANN was used to enhance Bs production by 70%.

ANN has been compared to the use of statistical design, and ANN has been shown to be more accurate in predicting the optimum conditions for BS production as in the case of the study carried out by Pal et al. [24]. These authors improved the Bs (glycolipid containing trehalose as the major carbohydrate) production in *Rhodococcus erythropolis*. Because glycolipids are predominantly associated with cell growth, the factors affecting growth were analyzed first. An organic nitrogen source favored biomass production, while inorganic sources showed poor growth. Sucrose and glucose resulted in the best biomass yields, while toluene, paraffin, olive oil, and castor oil were good Bs inducers. The factors selected for optimization with ANN and RSM were sucrose, yeast extract, meat peptone, and toluene. At the end of the optimization experiments, Bs production yield was increased by 3.5-fold.

8. Improving Downstream Processes

Although cost effective Bs production is the major priority for feasible industrial application, downstream processing should be taken into account. Once the secondary metabolite has been produced, the need for high purity of the tenso-active agent will depend on the application intended [52]. Recovery and purification has been shown to greatly increase production costs, especially in the case of proteins, and Bs are no exception [5]. Traditional strategies for Bs recovery and purification include precipitation with acids, salts, or organic solvents which are toxic and cause air pollution. Other more sophisticated methods include foam fractionation, ultrafiltration, adsorption-desorption on polystyrene resins, ion exchange chromatography, and High Pressure Liquid Chromatography (HPLC) [4,13]. However, the latter present very low yields, as only small amounts of the crude extract can be treated. A new approach which has recently gained popularity for downstream processing in other biomolecules is Aqueous Two-Phase Systems (ATPS). This strategy has been successful for the recovery and partial purification of enzymes and other micro and macro molecules, but has been hardly used for the treatment of crude Bs extracts [53]. Up to date, the application of ATPS for Bs downstream processing remains basically unexplored in the biotechnological field.

In fact, we found only one study dedicated to the use of statistical design for the improvement of Bs recovery [48]. They optimized the extraction conditions of a *Lactobacillus pentosus* cell bound biosurfactant/emulsifier using a Box–Behnken response surface methodology. Their results showed that operation time was the most influential independent variable, followed by temperature and salt concentration. The optimum extraction conditions of the biosurfactant were achieved at 45°C at 120 min and using 9 gkg^{-1} of salt. Bs yield improved from $9.49\text{--}13.76 \text{ mgL}^{-1}$. Their study serves as an excellent example for others in this field.

Researchers can and should exploit the possibilities for enhancing Bs yields after production optimization. Here we propose the use of designs such as the PBD for the screening of important factors

affecting Bs recovery, and subsequently, the implementation of other designs for determining the most adequate levels of the relevant factors such as solvent concentration, temperature, and exposition time.

9. Concluding Remarks

The use of statistical designs are not absolutely necessary for enhancing Bs production because the traditional methods are still valid. However, the use of statistical approaches confers various advantages, not only in the amount of experiments and determining the influence and effect on the factors analyzed and their interactions, but also, results in the enhancement of production yields. Additionally, in most cases, this approach makes production more cost effective. The application of one or more experimental designs strictly depends on the objective and ambition of the investigator/company. However, the microorganism of study and the factors to be evaluated should be analyzed and scientifically justified.

Funding: Research funded by the National Council of Science and Technology of Mexico—Secretariat of Energy—Hydrocarbon Trust, project 201441. This is a contribution of the Gulf of Mexico Research Consortium (CIGoM).

Conflicts of Interest: There are no conflicts of interest among authors.

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