Vol.67: e24220225, 2024
https://doi.org/10.1590/1678-4324-2024220225
ISSN 1678-4324 Online Edition

BABT
Brazilian Archives of Biology and Technology

*Article - Agriculture, Agribusiness and Biotechnology*

# Biosurfactant by *Serratia* sp. BR13816: Fermentation Optimization and Nanoemulsion Formation

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Editor-in-Chief: Paulo Vitor Farago
Associate Editor: Luiz Gustavo Lacerda

Received: 27-Mar-2022; Accepted: 02-Dec-2023

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**HIGHLIGHTS**

- Biosurfactant producer *Serratia* sp. BR13816 was isolated from Amazonian soils
- Experimental design approach was applied for optimization of medium components.
- Nanoemulsions with the cell-free crude extracts were prepared with success.

**Abstract:** The main objective of this study was to optimize the fermentation process using *Serratia* sp. BR13816, a bacterial isolate from Amazonian soil, and to evaluate the formation of nanoemulsions. Submerged fermentation was carried out with different carbon and nitrogen sources. Based on the emulsification index and surface tension results, we selected a hydrophobic (corn oil) and a hydrophilic (glycerol) carbon source, and a nitrogen one (urea). For fermentation using corn oil/urea greater emulsification indexes and lower surface tensions were verified at pH 8, 25 °C, and 48 h, whereas for glycerol/urea-supplemented medium the best conditions were attained at pH 7, 30 °C, and 96 h. These pre-selected factors (pH, temperature, and time) were used in a central composite rotatable design (CCRD) for an additional fermentation optimization of the two carbon/nitrogen mediums, corn oil/urea and glycerol/urea. The two best nutritional systems based on corn oil/urea and glycerol/urea were selected using a central composite rotatable design (CCRD). The mathematical models using the response surface methodology showed adequate adjustment. The best run of the experimental design for corn oil/urea (6.00% /0.60%) and glycerol/urea (3.00% /1.40%) systems presented surface tension values of 35.70 mN/m and 37.10 mN/m, respectively. The nanoemulsions produced by a low-energy method presented average sizes by dynamic light scattering varying from 453.1 nm to 667.3 nm when 0.1% of the oil was employed. Therefore, *Serratia* sp. BR13816 showed promising biosurfactant-producing potential for future industrial applications.

**Keywords:** Natural surfactant; *Serratia*; Surface Tension; Response surface methodology; Nanosystems.

## INTRODUCTION

Biosurfactants are metabolites produced under specific conditions by a wide variety of microorganisms. Such molecules present great structural diversity and are classified into glycolipids, lipopeptides, lipoproteins, phospholipids, fatty acids, polymeric, and particulate biosurfactants [1]. The main function of biosurfactants is to reduce the surface and interfacial tension of immiscible fluids, as in oil-water systems, allowing miscibility among components. Due to their structural diversity, biosurfactants play distinct functions and might be suitable for applications in the petrochemical, environmental, pharmaceutical, food, and agricultural sectors [2].

Biosurfactants present advantages over synthetic surfactants, such as low toxicity, being produced from renewable sources, greater degradability, and resistance to environmental conditions, among others [3,4]. They can also favor the formation of emulsions and nanoemulsions, providing different physicochemical, sensory, or biological attributes desirable to products, such as appearance, texture, and stability [5]. Thus, emulsions and nanoemulsions containing these types of molecules can be widely used in commercial products of the food, beverage, and cosmetics industries [5,6].

Despite the numerous advantages of biosurfactants, production costs are still a limitation for their industrial usage [7]. According to Reis and coauthors [4], two strategies may be adopted to reduce costs: (i) identification and use of microorganisms with enhanced production capacity, and (ii) optimization of the growth medium conditions. Several environmental factors influence biosurfactant yield and quality, particularly the source of carbon and nitrogen, pH, aeration, inoculum quantity, and incubation period [8]. Optimization of these factors may increase biosurfactant production by microorganisms [9]. The one variable at a time (OVAT) method is a widely used approach to optimize factors affecting metabolite production by microorganisms. Nonetheless, this methodology fails to assess interactions among the factors [10].

In order to overcome this limitation, several statistical methods can be jointly employed with the OVAT technique to improve biosurfactant production, such as the response surface methodology [11,12], multivariable linear regression (stepwise) [13,14], as well as mathematical modeling and prediction by artificial neural networks (ANN) [14,15]. The present study aimed to optimize nutritional and environmental factors for biosurfactant production by *Serratia* sp. BR13816, making possible the usage of low-cost and easily available hydrophilic or hydrophobic carbon sources, and to evaluate the biosurfactant crude extract's effects on nanoemulsion formation.

## MATERIAL AND METHODS

### Bacteria strain

*Serratia* sp. bacterium was isolated from the Várzea ecosystem in Ferreira Gomes City, Amapá. The strain (BR13816 code) was deposited at the Johanna Döbereiner Biological Resource Center (Embrapa Agrobiologia) and the gene sequence at the NCBI GenBank (MK156427 code). The isolate was also registered under the code A49223C on the National Genetic Heritage Management System (SISGEN), as recommended by the Brazilian Biodiversity Law (n° 13.123/2015).

The genomic DNA extraction was carried out using the Wizard® Genomic DNA kit (Promega, Madison, WI, USA), following the manufacturer's recommendations. DNA concentration was measured by spectrophotometry at 260 nm (NanoDrop, Thermo Fisher Scientific, Waltham, MA, USA), and their integrity was verified on agarose gel at 1% (w v-1; 60 V; 1 h).

The 16S rDNA gene was amplified with the primers 27F 5′-AGA GTT TGA TCC TGG CTC AG- 3′ and 1492R 5′-GGT TAC CTT GTT ACG ACT T-3′. The PCR reaction was realized on a thermal cycler (Applied Biosystems™ SimpliAmp) under the following conditions: 1.5 U Taq DNA polymerase, 1x PCR buffer (10 mM of Tris-HCl pH 8 and 50 mM of KCl), 1.75 mM of MgCl2, 0.25 mM of each dNTP, 0.2 µM of each primer, and 1 µL of the DNA template, with a total volume of 50 µL. Amplification was performed using initial denaturation at 94 °C for 3 min, followed by 29 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min, extension at 72 °C for 2 min, and a final extension at 72 °C for 7 min.

Sequencing reactions were carried out using a DYEnamic™ ET Dye Terminator kit (MegaBACE™) and an automatic MegaBACE 1000 sequencer (GE Healthcare Life Sciences). The obtained sequence was deposited at the NCBI GenBank with accession number MK156427. We compared the obtained sequence with the National Center Biotechnology Information database (http://www.ncbi.nlm.nih.gov) using the BLAST tool [16] and calculated their similarity with the type strain of described species belonging to the most closely related genus using a sequence identity matrix in the BioEdit software.

### Inoculum preparation

The microorganism was inoculated in nutrient agar plates and incubated at 30 °C for 24 h. The colonies were transferred to tubes using a platinum loop containing nutrient broth and incubated at 30 °C for 72 h. The fermentation tests used 1 mL of the inoculum, with approximately $10^8$ CFU/mL.

### Fermentation process

The biosurfactant production was conducted using a mineral salt medium (MSM) adapted from Fadhile Almansoory and coauthors [17]. The medium contained 1.2 g/L $KH_2PO4$, 1.8 g/L $K_2HPO_4$, 4.0 g/L $(NH_4)_2SO_4$, 0.2 g/L $MgSO_4.7H_2O$, 0.01 g/L $FeSO_4.7H_2O$, 0.1 g/L NaCl and 1 mL of trace elements. Trace elements' composition comprised 0.1 g/L $MnSO_4.H_2O$, 0.025 g/L $CuCl_2$, 0.025 g/L $(NH_4)_6Mo_7O_{24}.4H_2O$, 0.025 g/L $Co(NO_3)_2$, 0.025 g/L ZnCl and 0.01 g/L $(NH_4)_2SO_4$. The pH of the medium was adjusted using 1 mol/L sodium hydroxide (NaOH) and hydrochloric acid (HCl). The tests were performed using a 125 mL Erlenmeyer flask containing 100 mL of the culture medium and a stationary regime [18].

### Effects of carbon and nitrogen sources

The carbon sources used for biosurfactant production were sucrose (C1), fructose (C2), glycerol (C3), soy oil (C4), corn oil (C5), olive oil (C6), and residual soy oil (C7). The mineral medium was supplemented with 5.0% of the carbon source. After the selection of the best two carbon sources, four nitrogen sources were also explored by adding 0.5% of $(NH_4)_2SO_4$ (N1), peptone (N2), yeast extract (N3), and urea (N4) to the mineral medium. For all experiments, pH was adjusted to 7.0, and the cultures were incubated at 30 °C for 96 h.

### Effects of pH, temperature, and time of reaction

The effect of operational parameters was investigated for mineral mediums supplemented with 0.5% (w/v) of the best nitrogen source and 5.0% of one of the two best carbon sources. Initially, different pHs (5.0, 6.0, 7.0, 8.0, and 9.0) were tested for biosurfactant production at 30 °C for 96 h. Temperature variation (25, 30, 35, and 40 °C) was analyzed considering the selected pH and reaction time of 96 h. Finally, different times (48, 96, 144, and 192 h) were evaluated for fermentation reactions at the pH and temperature previously selected.

### Emulsification and surface tension tests

The culture media obtained from optimized conditions were centrifuged at 8064 g/10 min at 4 ºC to obtain a cell-free supernatant to determine the emulsification capacity and surface tension. The emulsification test was performed by adding 2.0 ml of commercial kerosene to a screw-cap test tube containing 2.0 ml of cell-free supernatant, followed by vigorous mixing in a vortex for 2 min [19]. Measurements were carried out at room temperature, and the emulsification index (E24h) was calculated by the ratio between emulsion column height after 24 h and total column height. Surface tension was measured by the ring Du Noüy method using a KRUSS (Easydyne) tensiometer, according to the methodology described by Kuyukina and coauthors [20]. The cell-free supernatant was added to a glass container, and the measurement was carried out by placing the ring on the surface for the equipment to read. Before each test, the DU NUOY ring was sterilized using a Bunsen burner and calibrated with distilled water (~ 70.4 ± 0.1 mN/m at 37 ºC), as proposed by Du Noüy [21]. The analyses were realized at room temperature and in triplicate.

### Fermentation optimization

After the preliminary analysis of the nutritional and operational parameters, central composite rotatable designs (CCRD) coupled with response surface methodology (RSM) were used to optimize fermentation (Table 1) using the best hydrophilic and hydrophobic carbon sources, glycerol and corn oil, respectively.

**Table 1.** Central composite rotatable design (CCRD) matrix of independent variables for the fermentation process using the best hydrophobic and hydrophilic carbon source.

| Run | Coded Variables | | Hydrophobic carbon-source (oil corn) containing system | | Hydrophilic carbon source (glycerol) containing system | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | First design | | Second design | |
| | Carbon source concentration | Nitrogen source concentration | Carbon (g/L) | Nitrogen (g/L) | Carbon (g/L) | Nitrogen (g/L) | Carbon (g/L) | Nitrogen (g/L) |
| 1 | -1 | -1 | 6.00 | 0.40 | 2.50 | 0.25 | 1.00 | 0.60 |
| 2 | +1 | -1 | 8.00 | 0.40 | 7.50 | 0.25 | 3.00 | 0.60 |
| 3 | -1 | +1 | 6.00 | 0.60 | 2.50 | 0.75 | 1.00 | 1.40 |
| 4 | +1 | +1 | 8.00 | 0.60 | 7.50 | 0.75 | 3.00 | 1.40 |
| 5 | -1.41 | 0 | 5.58 | 0.50 | 1.46 | 0.50 | 0.58 | 1.00 |
| 6 | +1.41 | 0 | 8.41 | 0.50 | 8.53 | 0.50 | 3.41 | 1.00 |
| 7 | 0 | -1.41 | 7.00 | 0.35 | 5.00 | 0.15 | 2.00 | 0.43 |
| 8 | 0 | +1.41 | 7.00 | 0.64 | 5.00 | 0.85 | 2.00 | 1.56 |
| 9 | 0 | 0 | 7.00 | 0.50 | 5.00 | 0.50 | 2.00 | 1.00 |
| 10 | 0 | 0 | 7.00 | 0.50 | 5.00 | 0.50 | 2.00 | 1.00 |
| 11 | 0 | 0 | 7.00 | 0.50 | 5.00 | 0.50 | 2.00 | 1.00 |
| 12 | 0 | 0 | 7.00 | 0.50 | 5.00 | 0.50 | 2.00 | 1.00 |

Each experimental design comprised two independent factors (carbon and nitrogen source concentrations), varying at five levels (-1.41, -1.0, 0, 1, 1.41), and four replicates of the central point, totaling 12 runs. For the system containing the corn oil, one experimental design using the selected carbon (5.58, 6.0, 7.0, 8.0, and 8.41) and nitrogen (0.35, 0.40, 0.50, 0.60, and 0.64) concentrations (%) was evaluated. For the system containing glycerol, a first design considering higher carbon levels (1.46, 2.5, 5.0, 7.5, 8.53) and lower nitrogen ones (0.15, 0.25, 0.5, 0.75, 0.85) was realized. Once this design did not provide a good mathematical model ($R^2$< 0,7), the variables were adjusted using the steepest descent optimization. The experiment was moved along the path in which the carbon source concentrations were decreased until the response did not reduce any longer. After this evaluation, new concentrations for the variables were chosen as the central point to optimize the medium using a second CCRD design. Therefore, the following carbon concentrations (0.58, 1.0, 2.0, 3.0, and 3.41) and nitrogen ones (0.43, 0.6, 1.00, 1.40, and 1.56) were used in the second design of the glycerol-containing medium. For all designs, the surface tension was adopted as the dependent variable once it is closely related to the biosurfactant yield. Experiments were conducted at pH, time, and temperature previously selected. All experimental designs were analyzed using the Minitab® 19 software. Experimental data were adjusted and represented by a second-order polynomial equation.

## Nanoemulsion preparation and characterization

Nanoemulsions (NE) were prepared using a low-energy method [22]. The 5 mL of the aqueous phase, composed of the biosurfactant crude extract (cell-free supernatant), was added dropwise to the oily phase (soy oil) under continuous homogenization using a vortex mixer (Kasvi®, model K40-10208). The oil concentrations tested were 0.1, 0.5, and 1% to produce NE with biosurfactant crude extracts obtained from corn oil (CO-NE) and glycerol (GL-NE) carbon sources. The hydrodynamic diameter and polydispersity (PdI) of the NE were determined by dynamic light scattering (DLS) analysis using the Zetasizer Nano ZS (Malvern Instruments®, United Kingdom) equipped with a 10 mW red laser (λ = 632.8 nm) and a 90° fixed-angle detector at 25 °C room temperature. The zeta potential (PZ) was measured on the same equipment by electrophoretic mobility. Samples were previously diluted with ultrapure water (1:25). Samples were evaluated in triplicate, and the results were expressed as the mean ± standard deviation. The best NE were stored at room temperature, and the stability was evaluated after 7 and 14 days.

### Statistical analysis

All obtained data were analyzed using a one-way analysis of variance (ANOVA) and means compared by the Tukey test at 5% significance using the Minitab® 19 software.

## RESULTS AND DISCUSSION

### Selection of carbon and nitrogen sources

Biosurfactant production is affected by different parameters such as microorganism strain, carbon sources, nitrogen sources, salt concentration, temperature, pH, agitation, and aeration [23]. Carbon supplementation is fundamental to enhancing the quantity and quality of the biosurfactant produced in the fermentative process. As shown in Figure 1, the *Serratia* isolate was able to grow in all tested carbon sources. Glycerol and sucrose resulted in higher emulsification index values (50.4 ± 1.3% and 51.1 ± 4.9%, respectively), but corn oil was the most effective in reducing the surface tension (40.5 ±0.3 mN/m).
Hydrophobic compounds' rich substrates, such as soybean, coconut fat, castor, corn, olive, and sunflower vegetable oils, have also been reported as suitable carbon sources for the production of biosurfactants by *Serratia* species [24]. For the production of prodigiosin-like pigment by *Serratia marcescens* SMΔR, the addition of vegetable oils (2–6% [v/v]) enhanced the fermentation broth markedly [25]. Ferraz and coauthors [24] reported that sunflower oil typically contains around 60% linoleic acid, which decreased the surface tension significantly, indicating that this fatty acid stimulated the production of biosurfactant by *Serratia marcescens* LB006. A bacteria of this species degraded *Cymbopogon martini* (palmarosa) green oil efficiently by using its carboxyl group as the carbon source [26]. Different hydrocarbons such as n-hexadecane, canola, sunflower, diesel, and engine oils also proved to induce high production of biosurfactants by *Paenibacillus* sp. D9 [27]. Among the hydrophobic carbon sources tested in the present study, corn oil was the most effective in favoring surface tension reduction; thus, it was selected for further investigation.

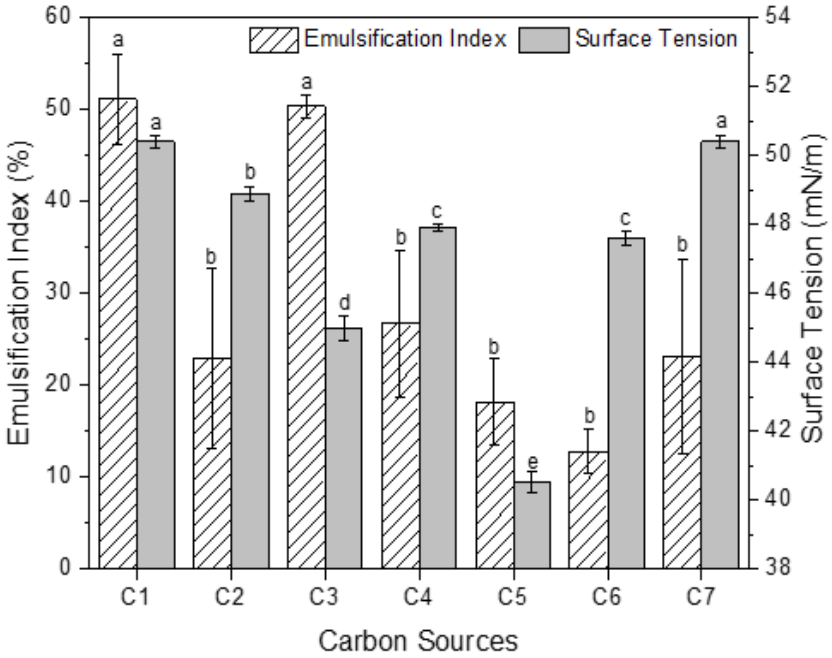
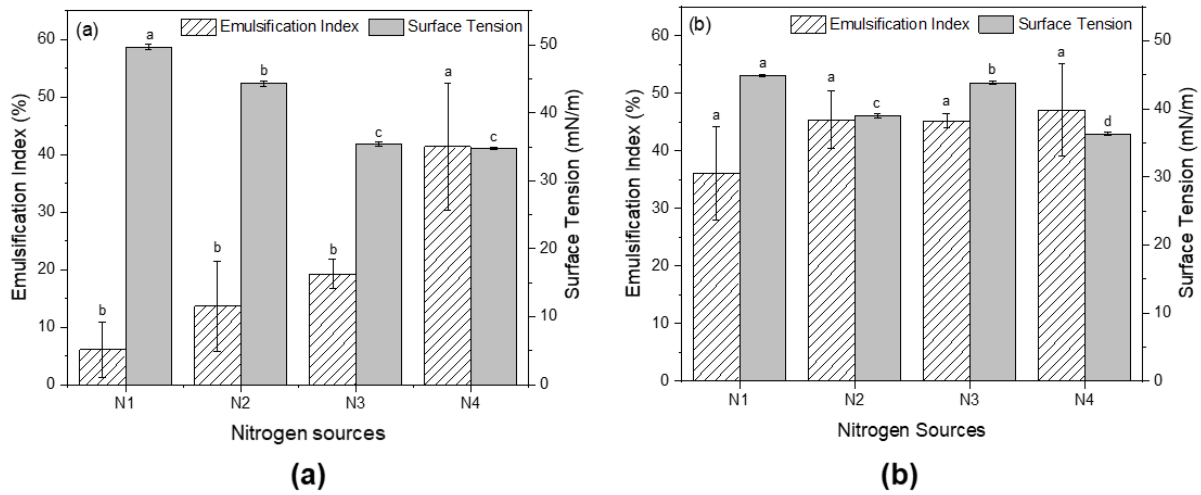



**Figure 1.** Effect of carbon sources on the emulsification index and surface tension. C1: sucrose; C2: fructose; C3: glycerol; C4: soy oil; C5: corn oil; C6: olive oil; and C7: residual soy oil. Means classified by the same case letter did not differ according to the Tukey test at 5% significance.

Hydrophilic carbon sources, such as glycerol, glucose, fructose, and sucrose, have been used for biosurfactant production by *Serratia* species [17]. The use of 2% (w/v) sucrose as a carbon source for the cultivation of *Serratia* MTCC86 favored biosurfactant production with outstanding emulsification activity and good surface properties [27]. Glycerol has also been indicated as a suitable substrate for the optimized biosurfactant production by *Serratia marcescens* [29]. Sucrose and glycerol, used as sole carbon sources, also proved to improve biosurfactant production by different *Bacillus* strains [30,31]. Fadhile Almansoory and coauthors [17] evaluated submerged fermentation by a *Serratia marcescens* strain using these two

substrates (glycerol and sucrose) and indicated that glycerol resulted in higher biosurfactant production and a greater reduction of surface tension. Figure 1 shows that sucrose and glycerol presented similar emulsification capacities, but the latter carbon source reduced the medium's surface tension more significantly.

As can be seen in Figure 2, urea was the best nitrogen source for fermentation by the *Serratia* strain, independent of the carbon source (Figures 2a, 2b). For corn oil-supplemented medium, yeast extract and urea resulted in a similar reduction in surface tension (35.4 ± 0.3 mN/m and 34.8 ± 0.3 mN/m, respectively), but urea produced a higher emulsification index. For the culture medium supplemented with glycerol, all tested nitrogen sources presented emulsification capacity statistically equivalent, but urea addition generated the lowest surface tension value (36.3 ± 0.3 mN/m).




**(a)** **(b)**

**Figure 2**. Effect of nitrogen sources on the emulsification index and surface tension when the fermentation medium is supplemented with corn oil (a) and glycerol (b). N1: $(NH_4)_2SO_4$; N2: peptone; N3: yeast extract; and N4: Urea. Means classified by the same case letter did not differ according to the Tukey test at 5% significance.

Nitrogen is the second most essential supplement for biosurfactant production by microorganisms. A significant number of nitrogen sources have already been reported in fermentative processes by *Serratia* species, such as peptone, yeast extract, ammonium sulfate, ammonium nitrate, and ammonium chloride, as well as some mixtures between these compounds [17,23]. For *Pseudomonas aeruginosa* MR01, yeast extract, urea, and peptone presented comparable influences on biosurfactant production [32]. *Serratia ureilytica* sp. nov. was able to hydrolyze urea to ammonia, using it as the sole nitrogen source for its growth [33]. For a *Serratia nematodiphila* strain, a 20% increase in biosurfactant levels was attained when urea was used as the nitrogen source [34]. An excellent biosurfactant yield and surface tension reduction were also attained by *Serratia marcescens* ZCF2 when olive oil was used as the carbon source and urea as the nitrogen one [35]. Therefore, as urea presented superior behavior when compared to the other tested nitrogen sources, it was chosen for additional evaluations using the selected carbon sources (corn oil and glycerol).

## Selection of pH, temperature, and reaction time

Physicochemical conditions can play an essential role in the biosurfactant production output of microorganisms. The *Serratia* strain grew and produced biosurfactants at a wide range of pHs, from 5.0 to 9.0 (Figure 3). When the growth medium was supplemented with corn oil/ urea (Figure 3a), a high emulsification index (48.9 ± 1.6%) and the lowest mean value of surface tension (36.1 ± 0.3 mN/m) occurred at pH 8.0. Surface tension results for experiments conducted at pH 5.0 and 9.0 were similar to the ones attained at pH 8.0, according to the Tukey test. Nonetheless, the emulsification capacity was impaired at pH 5, 7, and 9. For fermentation processes using glycerol/urea (Figure 3b), the emulsification index obtained from pH 6.0 to 8.0 was around 55%, but neutral pH resulted in the lowest surface tension (34.0 ± 0.2 mN/m).

Different strains of *Serratia marcescens* have been found to produce biosurfactants at pH ranging from 4 to 9 [17,29]. Jeon, Jung, and Park [36] found that *Serratia* sp. SK090424 cultivated in a mineral-based soybean oil medium showed significantly higher biosurfactant production at pH 8 than pH 7. According to these authors, the biosurfactant's increased activity at pH 8 might be a consequence of the greater emulsification of soybean oil under alkaline conditions, as bacterial cells may better absorb and metabolize

the emulsified oil. For mineral medium supplemented with glycerol or other hydrophilic carbon sources, pH 7.0 or pH 7.2 is broadly cited as an optimal condition for biosurfactant production [23].

Once biosurfactant production generally occurs through several enzymatic reactions inside the cells [37], pH may impair their activities [38] and, consequently, the microorganism's metabolism. Considering these aspects, pH 8.0 and pH 7.0 were selected for fermentation using corn oil/urea and glycerol/urea-containing systems, respectively.

The influence of temperature on fermentation processes using corn oil/urea at pH 8.0 and glycerol/urea at pH 7.0 can be seen in Figure 4. For the first one, the lowest surface tension (31.8 ± 0.3 mN/m) was verified after incubation at 25 °C. The emulsification index did not show significant differences in the range of 25 °C to 35 °C but dropped markedly at 40 °C. When the medium was supplemented with glycerol/urea at pH 7.0 (Figure 4b), the emulsification was inhibited at 25 °C, and a comparatively low value was also obtained at 40 °C. Fermentation processes conducted at 30 °C and 35 °C demonstrated statistically equivalent results and similar surface tension mean values (around 37 mN/m).

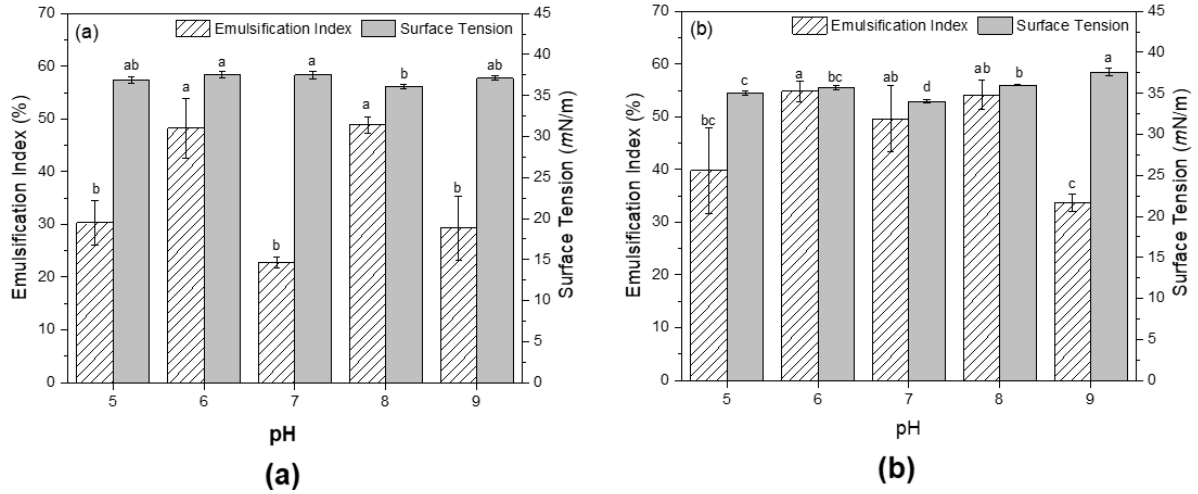



**Figure 3.** Effect of pH on the emulsification index and surface tension for the biosurfactant production using (a) corn oil and urea, and (b) glycerol and urea. Means classified by the same case letter did not differ according to the Tukey test at 5% significance.

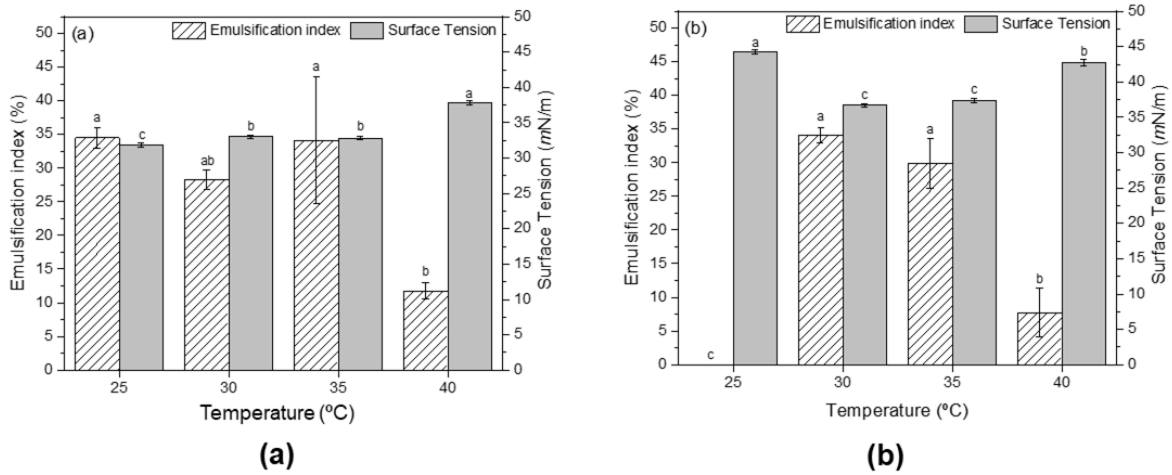



**Figure 4.** Effect of temperature on the emulsification index and surface tension for the biosurfactant production using (a) corn oil and urea at pH 8, and (b) glycerol and urea at pH 7. Means classified by the same case letter did not differ according to the Tukey test at 5% significance.

The selection of the ideal incubation temperature is essential in fermentation processes as it may also affect enzymatic reactions involved in the microorganisms' metabolism [38]. For *Serratia marcescens* DT-1P, a temperature of 26.6 °C was reported as optimum [29]. In another work, the temperature of 30 °C was the best condition for biosurfactant production by *Serratia* strains when glycerol and olive oil-enriched mediums were employed [17,35]. *Serratia marcescens* 274 showed temperature-dependent activity, as the red pigment prodigiosin and the biosurfactant serrawettin were produced at 30 °C but not at 37 °C [39]. Here,

the temperatures of 25 °C and 30 °C were chosen for the fermentation using corn oil/urea and glycerol/urea-containing systems, respectively, which is in agreement with the literature.

A time from 48 to 192 h was also evaluated to optimize fermentation (Figure 5). It can be seen the highest emulsification index and lowest surface tension until 96 h when fermentation was conducted at pH 8 and 25°C using the corn oil/urea-supplemented medium (Figure 5a). After 96 h, these parameters decreased significantly. For corn glycerol/urea-supplemented medium culture at pH 7 and 30 °C, the highest emulsification index and the lowest surface tension were observed after 96 h (34.6 ± 0.2 mN/m).

The production of surface-active compounds in culture broth is a time-dependent process [18]. After the critical micellar concentration, biosurfactant production begins to increase at significantly lower rates, and no further reduction in surface tension is observed [24]. Probably, the critical micellar concentration was reached for both investigated systems (Figure 5), leading to a significant drop in the biosurfactant production rates. Consequently, no further decrease in surface tension was verified. In the case of the corn oil/urea system, the emulsification ability of the biosurfactant may have been negatively influenced by the production of other secondary metabolites.

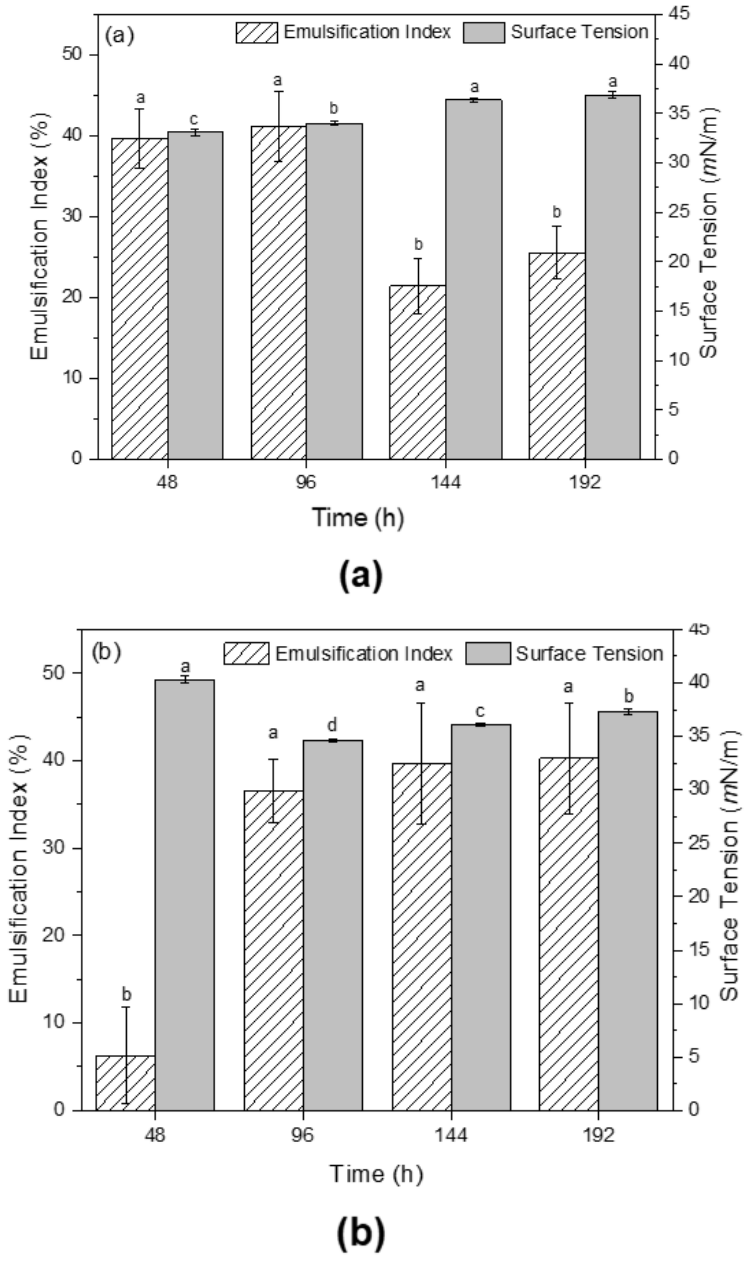



**Figure 5.** Effect of fermentation time on the emulsification index and surface tension for the biosurfactant production using (a) corn oil and urea at pH 8 and 25 °C, and (b) glycerol and urea at pH 7 and 30 °C. Means classified by the same case letter did not differ according to the Tukey test at 5% significance.

For *Serratia marcescens* MTCC, maximum surface tension reduction coincided with maximum biosurfactant production and was observed after 24h [28]. Similar behavior was also reported by Fadhile Almansoory and coauthors [17] for a *Serratia marcescens* strain but after 120 h. The optimal cultivation time for *Serratia* species is strongly dependent on strain, growth medium, and growth conditions and can vary from 24 h to 168 h [23]. Based on previous aspects, the incubation times of 48 h and 96 h, which are in agreement with the literature, were selected for the fermentation processes using the corn oil/urea (at pH 8 and 25 °C) and the glycerol/urea (at pH 7 and 30 °C) enriched mediums, respectively.

## Optimization of carbon and nitrogen source concentrations

Central composite rotatable designs were used to investigate optimal carbon and nitrogen concentrations for biosurfactant production based on surface tension measurements. Experimental designs were previously presented in the methodology section (Table 1), whereas responses can be seen in Table 2. Fermentation processes were conducted using optimized operation parameters for corn oil/urea (pH 8, 25 °C, and 48 h) and glycerol/urea-supplemented mediums (pH 7, 30 °C, and 96 h). ANOVA results for the experimental design of the enriched medium are shown in Table 3.

**Table 2.** Experimental and predicted surface tension values of the cell-free supernatant obtained in the fermentation process using corn oil and glycerol as carbon sources and urea as the nitrogen one.

| Run | Surface Tension (*m*N/m) | | | | | |
|---|---|---|---|---|---|---|
| | Corn oil/urea system | | Glycerol/urea system | | | |
| | | | First design | | Second design | |
| | Observed | Predicted | Observed | Predicted | Observed | Predicted |
| 1 | 43.4 ± 0.1 | 44.4 | 37.3 ± 0.5 | 37.5 | 39.0 ± 0.4 | 39.2 |
| 2 | 48.0 ± 0.5 | 45.0 | 38.1 ± 0.3 | 38.7 | 38.5 ± 0.3 | 38.2 |
| 3 | 35.7 ± 0.4 | 38.4 | 37.1 ± 0.4 | 37.5 | 39.8 ± 0.5 | 40.3 |
| 4 | 50.5 ± 0.5 | 49.2 | 39.5 ± 0.4 | 40.4 | 37.1 ± 0.3 | 37.2 |
| 5 | 43.5 ± 0.3 | 40.8 | 37.0 ± 0.4 | 36.8 | 40.8 ± 0.4 | 40.4 |
| 6 | 45.8 ± 0.4 | 48.8 | 40.6 ± 0.5 | 39.7 | 37.3 ± 0.3 | 37.5 |
| 7 | 42.9 ± 0.4 | 44.3 | 38.5 ± 0.4 | 38.2 | 38.4 ± 0.4 | 38.5 |
| 8 | 44.0 ± 0.5 | 43.0 | 40.1±0.5 | 38.4 | 38.9 ± 0.5 | 38.5 |
| 9 | 39.1 ± 0.5 | 38.8 | 39.4±0.4 | 38.5 | 38.3 ± 0.4 | 38.4 |
| 10 | 38.5 ± 0.6 | 38.8 | 38.7±0.4 | 38.5 | 38.4 ± 0.5 | 38.4 |
| 11 | 38.9 ± 0.4 | 38.8 | 38.4±0.5 | 38.5 | 38.1 ± 0.4 | 38.4 |
| 12 | 38.7 ± 0.4 | 38.8 | 37.3±0.3 | 38.5 | 38.6 ± 0.4 | 38.4 |

**Table 3.** ANOVA of the quadratic model for surface tension.

| Source | DF | PC | Adj. SS | Adj. MS | F-value | P-value |
|---|---|---|---|---|---|---|
| ***Design of the corn oil/urea system*** | | | | | | |
| Model | 5 | 81.59% | 171.735 | 34.3470 | 5.32 | 0.033 |
| Linear | 2 | 31.26% | 65.803 | 32.9016 | 5.10 | 0.051 |
| C (Corn oil) | 1 | 30.48% | 64.143 | 64.1431 | 9.93 | 0.020 |
| U (Glycerol) | 1 | 0.79% | 1.660 | 1.6602 | 0.26 | 0.630 |
| Square | 2 | 37.97% | 79.922 | 39.9608 | 6.19 | 0.035 |
| C*C | 1 | 20.27% | 58.081 | 58.0810 | 9.00 | 0.024 |
| U*U | 1 | 17.70% | 37.249 | 37.2490 | 5.77 | 0.053 |
| 2-Way Interaction | 1 | 12.36% | 26.010 | 26.0100 | 4.03 | 0.092 |
| C*U | 1 | 12.36% | 26.010 | 26.0100 | 4.03 | 0.092 |
| Error | 6 | 18.41% | 38.742 | 6.4570 | - | - |
| Lack-of-Fit | 3 | 18.31% | 38.542 | 12.8473 | 192.71 | 0.001 |
| Pure Error | 3 | 0.10% | 0.200 | 0.0667 | - | - |
| **Total** | 11 | 100.00% | - | - | - | - |

**Cont, Table 3**

| | | | | | | |
|---|---|---|---|---|---|---|
| ***First design of the glycerol/urea system*** | | | | | | |
| Model | 5 | 68.39% | 9.58132 | 1.91626 | 2.49 | 0.149 |
| Linear | 2 | 62.76% | 9.13465 | 4.56733 | 5.94 | 0.038 |
| Glycerol (G) | 1 | 53.44% | 6.64514 | 6.64514 | 8.64 | 0.026 |
| Urea (U) | 1 | 9.32% | 2.48951 | 2.48951 | 3.24 | 0.122 |
| Square | 2 | 1.65% | 0.35667 | 0.17833 | 0.23 | 0.800 |
| G*G | 1 | 0.60% | 0.00400 | 0.00400 | 0.01 | 0.945 |
| U*U | 1 | 1.05% | 0.32400 | 0.32400 | 0.42 | 0.540 |
| 2-Way Interaction | 1 | 3.98% | 0.09000 | 0.09000 | 0.12 | 0.744 |
| GL*UR | 1 | 3.98% | 0.09000 | 0.09000 | 0.12 | 0.744 |
| Error | 6 | 31.61% | 4.61535 | 0.76922 | - | - |
| Lack-of-Fit | 3 | 17.37% | 2.32535 | 0.77512 | 1.02 | 0.495 |
| Pure Error | 3 | 14.24% | 2.29000 | 0.76333 | - | - |
| **Total** | 11 | 100.00% | - | - | - | - |
| ***Second design of the glycerol/urea system*** | | | | | | |
| Model | 5 | 92.19% | 10.0487 | 2.00975 | 14.17 | 0.003 |
| Linear | 2 | 76.18% | 8.3037 | 4.15187 | 29.26 | 0.001 |
| Glycerol (G) | 1 | 76.17% | 8.3023 | 8.30230 | 58.52 | 0.000 |
| Urea (U) | 1 | 0.01% | 0.0014 | 0.00143 | 0.01 | 0.923 |
| Square | 2 | 4.91% | 0.5350 | 0.26750 | 1.89 | 0.232 |
| G*G | 1 | 4.46% | 0.5290 | 0.52900 | 3.73 | 0.102 |
| U*U | 1 | 0.45% | 0.0490 | 0.04900 | 0.35 | 0.578 |
| 2-Way Interaction | 1 | 11.10% | 1.2100 | 1.21000 | 8.53 | 0.027 |
| GL*UR | 1 | 11.10% | 1.2100 | 1.21000 | 8.53 | 0.027 |
| Error | 6 | 7.81% | 0.8513 | 0.14188 | - | - |
| Lack-of-Fit | 3 | 6.62% | 0.7213 | 0.24042 | 5.55 | 0.097 |
| Pure Error | 3 | 1.19% | 0.1300 | 0.04333 | - | - |
| **Total** | 11 | 100.00% | 10.9000 | - | - | - |

DF: Degree of freedom; PC: percentage contribution; SS: Sum of squares; MS: Mean square.

ANOVA data (Table 3) indicated that the model for corn oil/urea was significant (p-value: 0.033) and predictable (F-value: 5.32), whereas the one for the glycerol/urea system did not show significance at a 5% level and did not provide a good mathematical adjustment ($R^2 < 0,7$). In order to achieve an adequate model for the glycerol/urea system, variables were adjusted using a steepest descent optimization (40). Five runs of the first experimental design were selected, as indicated in Table 4.

**Table 4.** Steepest descent experiment design and response values.

| Trials (first design) | Glycerol | Urea | Surface tension (mN/m) |
|---|---|---|---|
| 1 | 8.53 | 0.50 | 40.6 |
| 2 | 7.50 | 0.75 | 39.5 |
| 3 | 5.00 | 0.85 | 40.1 |
| 4 | 2.50 | 0.75 | 37.1 |
| 5 | 1.46 | 0.50 | 37.0 |

The mathematical model obtained to represent the surface tension (ST) as a function of the carbon and nitrogen concentrations (%) for the corn oil/urea system and the glycerol/urea one (the second experimental design) with their respective coefficients is given by Equations (1) and (2), respectively:

$$ST = 318.4 – 52.1\ C – 424U + 3.01\ C^2 + 241\ U^2 + 25.5\ C*U \quad (1)$$

$$ST= 39.30 – 0.794\ G + 1.69\ U + 0.287\ G^2 + 0.547\ U^2 – 1.375\ G*U \quad (2)$$

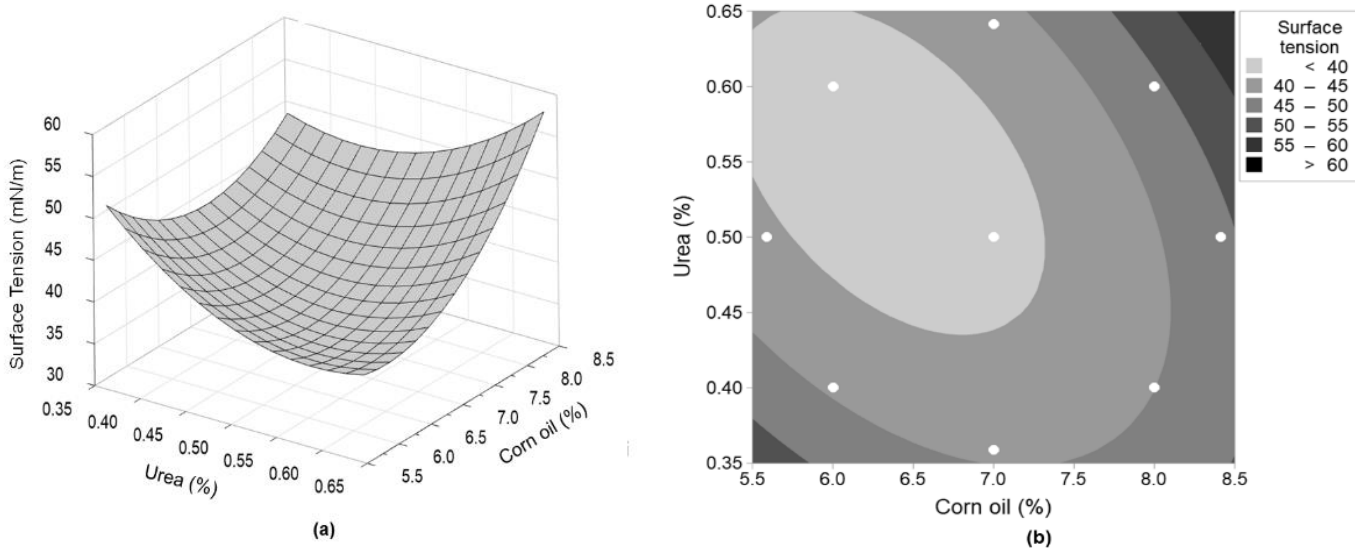
Where C, G, and U are the corn oil, glycerol, and urea concentrations, respectively. The coefficient of determination ($R^2$) was 81.50% and 92.19% for the corn oil/urea and glycerol/urea mediums, respectively.

Therefore, the regression model significantly explained the variation in response values. The most significant parameter in multiple regression analyses was the one with the lowest p-value and the largest t-value [41]. For the corn oil/urea system, the major contribution to the surface tension response was originated by the C term (p-value: 0.020; t-value: 3.15), followed by C2 (p-value: 0,024; t-value: 3.00), and U2 (p-value: 0,053; t-value: 2.40), indicating the highest contribution of second-order terms. In the case of the glycerol/urea medium (second design), the G term dominated the surface tension response, mainly due to its first-order contribution (p-value: 0.000; t-value: -7.65), but the interaction factor G*U also played a significant role (p-value: 0.027; t-value: -2.92). These results suggested that the studied variables (corn oil, glycerol, and urea concentrations) affected the surface tension in fermentation processes considerably.

The optimization goal was to obtain the minimum surface tension value for *Serratia* cultivation using hydrophobic and hydrophilic mediums, adjusting carbon and nitrogen concentrations in the studied range. The 3D response surface and contour maps for the mathematical models are presented in Figure 6. For the corn oil/urea system, the surface plot appears moderately concave (Figure 6a). The increase in corn oil or urea concentrations initially reduced surface tension but then raised it, showing a distorted parabolic trend for both parameters. The contour map exhibited regions of different surface tension ranges separated by concentric ellipses (Figure 6b). The third run of the experimental design (6.00% corn oil and 0.60% urea) dropped within the elliptic region which presents a surface tension lower than 40 mN/m. The response surface optimization indicated that 6.33% corn oil and 0.54% urea would further reduce surface tension. For the glycerol/urea-supplemented medium, a 3D plot showed a slightly deformed plane, in which the increase of urea and glycerol concentrations (in the studied range) caused a surface tension drop (Figure 6c). The contour plot revealed a lower surface tension area (< 37mN/m), which may be part of an elliptic region at the bottom of a concave 3D surface (Figure 6d). The fourth run (3.00% glycerol and 1.40% urea) of the experimental design fell very close to this area, but the optimized response (according to the regression model) would be attained using 3.41% of glycerol and 1.57% of urea.

After evaluation, a second experimental design to optimize this medium was performed, using a central point of 2% glycerol and 1% urea (Table 2). In this second experimental design for glycerol-urea-enriched medium, the fourth run (3.00% glycerol and 1.40% urea) was more effective in reducing surface tension (37.1 ± 0.3 mN/m). The regression model also achieved good agreement between the observed and predicted surface tension values, and ANOVA results show significance (p-value: 0.003 and F-value: 14.17) at a 5% level (Table 3).

Mathlom, Hayder, and Mahmood [42] tested different C:N ratios (6:1, 10:1, 13.3:1, 16:1, 20:1, and 30:1) in the mineral salt medium and indicated 20:1 as the optimal ratio condition for biosurfactant production by a *Serratia* strain. A Box-Behnken design used to evaluate the nutrient ratios' role on biosurfactant production by *Serratia marcescens* indicated that C/N = 5, C/Fe = 26,000, and C/Mg = 30 was the optimal condition to reduce the surface tension [43]. When used in the wastewater purification process, *Serratia marcescens* W5 showed efficient ammonium removal abilities in a wide range of C/N ratios at pH 7 [42]. The carbon-to-nitrogen ratio is of utmost importance in biosurfactant production, but it tightly depends on the supplement source and producer strain [22,45].



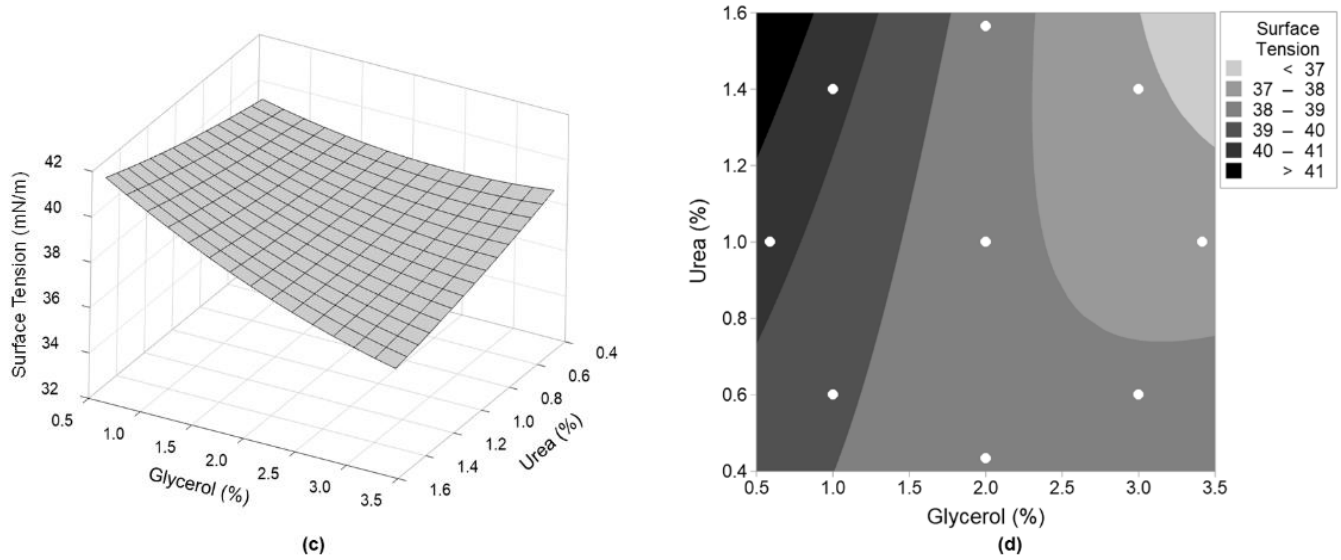



**Figure 6.** Three-dimensional surface and contour maps of the effect of carbon and nitrogen sources concentrations on surface tension. (a-b) corn oil/urea system; (c-d) Glycerol/urea system. Points marked in white in the contour plot indicated the runs of the experimental design.

A promising biosurfactant-producing microorganism must be able to decrease the growth medium's surface tension at least by 20 mN/m when compared with distilled water [46]. The third run of the experimental design for the corn oil/urea system and the fourth run of the glycerol one (second design) resulted in surface tension values of 48.8% (from 69.7 to 35.7) and 46.2% (from 68.9 to 37.1) lower than the used aqueous medium, respectively (Table 2). Pruthi and Cameotra [28] optimized biosurfactant production by *Serratia marcescens* MTCC 86 and found a maximum surface tension reduction (68 mN/m to 27 mN/m) corresponding to 60.3%, whereas Roldán-Carrillo and coauthors [43] attained a 50% drop (66 mN/m to 33 mN/m) for a different *Serratia* strain. Other works also suggested surface tension reductions of approximately 40% and 70% [47,48]. Therefore, the surface tension drop obtained for corn oil/urea and glycerol/urea-supplemented mediums suggested a suitable optimization.

Surface tension values seen after optimization of carbon and nitrogen source concentrations are slightly greater than some values observed in the previous section. Such results did not indicate incoherent surface tension values or problems in the optimization procedure but were a consequence of the methodology adopted for inoculum preparation. The fermentation tests were conducted using 1 mL of the inoculum, with approximately $10^8$ CFU/mL. Probably, the inoculum volume used for the optimization experiment presented a higher bacteria concentration, resulting in competition for the carbon source and its immediate consumption at the beginning of the fermentation process. Such a phenomenon would prevent the production of larger biosurfactant amounts and, consequently, surface tension reduction. Therefore, greater surface tension reduction might be achieved by the adjusting microorganism concentration in the inoculum for the optimized conditions.

## Effect of biosurfactant crude extracts in nanoemulsions (NE)

The droplet size distribution and zeta potential of the NE produced with the cell-free crude extracts (cell-free supernatant) obtained from corn oil/urea and glycerol/urea-enriched mediums containing the biosurfactant are presented in Table 5. All formulations showed fine and translucent aspects and a bluish reflectivity, according to the literature [22]. There was a significant difference among the hydrodynamic diameters ($p > 0.05$) of CO-NE and GL-NE produced with 0.1 and 0.5% of oil, indicating that the size was affected by the increase in oil concentration. At higher concentrations of oil, the sizes were larger, and no difference was seen between 0.5 and 1% of oil. The broad droplet size distribution observed can be attributed to a high concentration of non-dispersed emulsion droplets. However, it can be observed that NE produced with 0.1% of oil obtained the lowest mean droplet sizes, demonstrating that biosurfactants presented in both media had a limit of oil dispersion ($p<0.05$). CO-NE produced with 0.1% of oil presented the lowest mean size (453.1 nm) followed by GL-NE (667.3 nm), indicating that the biosurfactants presented in CO were more efficient than those presented in GL. This is following the lowest surface tension values previously obtained for corn oil/urea extract (35.70 mN/m).

**Table 5.** Physicochemical characteristics of nanoemulsions.

| Nanoemulsions | Average Particle Size ± SD* (nm) | Polydispersity Index ± SD* | Zeta Potential ± SD* (mV) |
|---|---|---|---|
| CO-01 | $453.10^{b}$ ± 10.69 | $0.594^{b}$ ± 0.002 | $-47.5^{a}$ ± 1.0 |
| CO-05 | $1004.47^{a}$ ± 174.61 | $0.765^{a}$ ± 0.033 | $-47.5^{a}$ ± 0.8 |
| CO-1 | $791.90^{a}$ ± 153.99 | $0.745^{a}$ ± 0.032 | $-47.0^{a}$ ± 0.8 |
| GL-01 | $667.43^{b}$ ± 41.52 | $0.532^{a}$ ± 0.123 | $-44.3^{a}$ ± 1.3 |
| GL-05 | $930.90^{a}$ ± 49.80 | $0.628^{a}$ ± 0.057 | $-45.7^{a}$ ± 1.2 |
| GL-1 | $926.97^{a}$ ± 93.70 | $0.660^{a}$ ± 0.084 | $-45.4^{a}$ ± 0.8 |

*SD: Standard deviation. Values followed by the same case letters in the same column did not differ from each other by the Tukey test at 5% significance

The PDI above 0.3 for all NE indicates polydisperse systems, and differences between CO-NE produced with 0.1 and 0.5% of oily phase were seen ($p<0.05$). All NE had negative surface charges, ranging from −44 to −47 mV. Values above 30 mV (in modulus) provide electrostatic repulsion between NE that contributes to stability [49]. As CO-NE and GL-NE produced with 0.1% of oil presented the best physicochemical characteristics, they were selected for stability evaluation during storage at room temperature until the 14th day (Figure 7).

The parameters evaluated at different times were the size, polydispersity index, and zeta potential. Both NEs presented size distribution stability until 7 days of storage. However, there was a significant increase in size distribution parameters after 14 days in comparison with the first day. The PDI remained above 0.3, indicating a polydispersed character. Zeta potential values decreased for both formulations but remained above 30 mV (in modulus).

Ostertag [50] demonstrated NE sizes of 930 nm and 140 nm when produced with the synthetic surfactants Tween 20 and Tween 80, respectively, by a low-energy method. They used 10% of oil (medium-chain triglycerides) and 25% of surfactant, values higher than described in this work. Despite the absence of reports about NE production with biosurfactants using low-energy methods in the literature, few works reported their production with high-energy ones. Sedaghat Doost and coauthors [51] produced a thymol nanoemulsion by a high-energy method employing 4% thymol and 1% of the biosurfactant Quillaja Saponin. They obtained NE with a mean droplet diameter varying from 245 to 166 nm and a PDI ranging from 0.48 to 0.30.

NE produced here presented a size distribution larger than those obtained in work with biosurfactants cited above. However, contrary to the reported works, here we employed the whole extract containing diverse substances, among them the biosurfactants.

The presence of bacterial metabolites could have hindered the oil droplet formation and/or the DLS measurement. Also, the yield of biosurfactant production by *Serratia* strains or other microorganisms can be lower than 0.1% (w/v) even in optimized conditions [23,52]. Therefore, NE sizes above 300 nm and the physical instability of CO-NE and GL-NE during storage may be attributed to the low biosurfactant concentration in the media when compared to the minimum of 1% of isolated biosurfactant employed in the literature. In addition, it is reported in the literature that the surfactant type and surfactant-to-oil ratio play an important role in NE size reduction and stability [50].

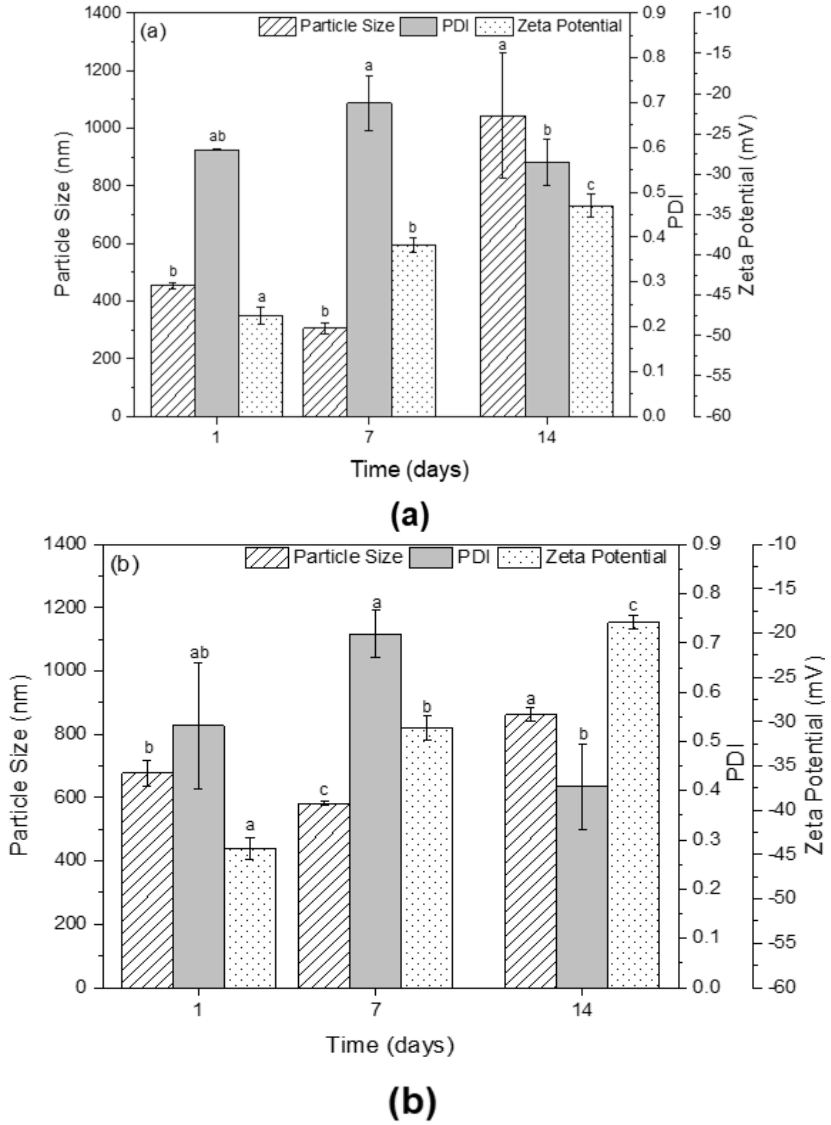



**Figure 7.** Particle size, polydispersity index, and zeta potential as a function of time for the nanoemulsions containing the biosurfactant crude extracts produced with (a) corn oil and urea (CO-01). and (b) glycerol and urea (GL-01). Means classified by the same case letter did not differ according to the Tukey test at 5% significance.

These results indicate that the biosurfactant produced by *Serratia* sp. bacteria isolated from Amazonian soil was able to produce nanoemulsions by a low-energy method that is easy to handle, has no organic solvent, and has a low energetic cost. Some parameters of the biosurfactant and NE process production could be modified to obtain smaller and more stable NE. Therefore, we contributed to the first report of NE based on biosurfactants produced by Amazonian-isolated bacteria.

## CONCLUSION

The present study successfully selected carbon and nitrogen sources as well as other growth parameters for biosurfactant production by *Serratia* sp. BR13816. Evaluation of the emulsification index and surface tension allowed the selection of a hydrophobic (corn oil) and a hydrophilic (glycerol) carbon source, as well as a nitrogen one (urea). For the fermentation medium supplemented with corn oil/urea, pre-selected operational parameters were pH 8, 25 °C, and 48 h, whereas for glycerol/urea were pH 7, 30 °C, and 96 h. The response surface methodology allowed an additional optimization of carbon and nitrogen concentrations, generating mathematical models with suitable adjustments. The lowest surface tension measured for corn oil/urea and glycerol/urea-supplemented mediums was 35.70 mN/m and 37.10 mN/m, respectively. Nanoemulsions produced with the cell-free crude extracts were prepared successfully by a low-energy method. The formulations containing 0.1% of oil generated the smallest particle size. Overall, our results demonstrated the potential of cell-free crude extracts obtained by *Serratia* sp. BR13816 isolated from Amazonian soil as an effective source of natural surfactants.

**Funding:** This work received financial support from the State University of Amapá (UEAP) and Fundação de Amparo à Pesquisa do Estado do Amapá (Process N°: 250.203.133/2018).
**Conflicts of Interest:** The authors declare no conflict of interest.

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