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Optimization of the Operational Conditions to Produce Extracellular and Cell-Bound Biosurfactants by *Aneurinibacillus aneurinilyticus* Using Corn Steep Liquor as a Unique Source of Nutrients

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Abstract: The relevance of this work lies in the fact that it is the first time that corn steep liquor (CSL) has been proposed as a unique source of nutrients for producing biosurfactants in a controlled fermentation and *Aneurinibacillus aneurinilyticus*, isolated from CSL, has been evaluated for producing extracellular and cell-bound biosurfactants in a controlled fermentation, using secondary raw materials as a source of nutrients. In the present study, *A. aneurinilyticus* was inoculated into the culture medium containing sterilized CSL solutions (100–400 g L^{-1}) and incubated using different temperatures (20–60 °C) and fermentation times (8–30 days). The dependent variables under study were the concentration of extracellular biosurfactants and cell-bound biosurfactant production in terms of critical micellar concentration (CMC), as well as the C/N ratio for cell-bound biosurfactant extracts. It was observed that CSL could increase the concentration of extracellular biosurfactants produced by A. *aneurinilyticus* if these were fermented during 19 days at 40 °C, using 250 g L^{-1} of CSL; a mean value of 6 g L^{-1} for extracellular biosurfactants and favorable CMC concentrations enabled the detection of cell-bound biosurfactant extracts under these conditions. Hence, these conditions could be considered optimal for producing both extracellular and cell-bound biosurfactants from CSL.

Keywords: corn; wet milling; secondary streams; sporulated bacillus; biosurfactants

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1. Introduction

Biosurfactants are surface-active compounds with properties similar to chemically synthesized surfactants. Because biosurfactants are composed of natural polymers containing lipids, sugars, or peptides, biologically produced surfactants have the advantage over their chemical synthetic analogs in terms of biodegradability and biocompatibility [1–3]. However, the major drawback of the production and commercialization of biosurfactants, when compared with chemical surfactants, is their high production costs, which are related to biotechnological production processes [4–7]. Therefore, recently, many studies have been focused on the utilization of low-cost carbon sources and nutrients, mainly residual streams, to produce more cost-competitive biosurfactants [8–15]. Kumar et al. [16] evaluated orange peel for lipopeptide biosurfactant production by *Bacillus licheniformis* under controlled growth conditions and detected maximum biosurfactant concentration (1.8 g L^{-1}) using 4% (*w*/*v*) orange residues at 40 °C. Gurjar & Sengupta [17] used a rice mill polishing residue to produce surfactin by *Bacillus subtilis* with a yield of 4.17 g kg^{-1} of residue. Recently, the use of corn steep liquor (CSL) was proposed as a direct source of biosurfactants by some authors [18–20]. Hence, a biosurfactant extract composed from lipopeptides, antioxidants, and phospholipids was extracted from commercial CSL [21]. The lipopeptide detected in the extract was composed from a series of amino acids, such as glutamic acid or glutamine,

aspartic acid or asparagine, glycine, alanine, arginine, proline, and leucine or isoleucine, whereas the fatty acid chain was constituted by C16 or C18 fatty acids.

Another biosurfactant extract was obtained from the strain of *Brevibacillus brevis* isolated from textile sludge [22]. The chemical analysis of this extract revealed the presence of lipopeptides composed of stearic acid and amino acids, including alanine, glycine, leucine, serin, proline, and asparagine. Recently, a microorganism was discovered in CSL, *Aneurinibacillus aneurinilyticus* (*A. aneurinilyticus*), which formed endospores and possessed the ability to produce cell-bound and extracellular biosurfactants [23].

Moreover, some studies performed with animal models have confirmed some probiotic properties of *A. aneurinilyticus* with a positive effect on growth and the immunological response of host organisms against pathogens [24,25]. However, the production of surface-active compounds by *A. aneurinilyticus* has scarcely been studied [23,26,27]. Balan et al. [27] proposed the centrifugation of fermented media to remove the cell biomass and obtain a cell-free supernatant which was subjected to acid precipitation at pH 2 using 6 N HCl. This method is different from others proposed in the literature. Hence, López-Prieto et al. [23] obtained cell-bound and extracellular biosurfactants by using liquid–liquid extraction or solid–liquid extraction, respectively, whereas Alenezi et al. [26] have focused mainly on the production of Gramicidin.

Recently, some authors have revealed the importance of CSL as a green biological resource of numerous bioactive compounds for the bioindustry [28]. One of the advantages of liquor is that the commercial CSL is subject to more exhaustive controls than other secondary raw materials proposed in the literature to obtain bioactive compounds. Moreover, Hull et al. [29] analyzed four different industrial processes for CSL in the corn milling industry at different steeping stages, observing a similar composition in amino acids with the prevalence of glutamine, glutamic acid, leucine, proline, aspartic acid, and asparagine. They also found a similar composition regarding simple and complex sugars and minerals between lots. These authors found some differences regarding the concentration of bioactive compounds from a quantitative point of view, probably because they compared CSL with different concentrations of solids. Moreover, the CSLs under evaluation were not subject to a stabilization phase where CSL was concentrated by up to 50% in solids, usually through the evaporation of water. Moreover, Martínez-Arcos et al. [30] analyzed CSL from FeedStimulants (50% in solids), corroborating the presence of minerals, amino acids (in equivalents of proteins), sugars, and organic acids in consonance with the typical composition of CSL. Hence, it would be interesting to increase our knowledge of the conditions that stimulate biosurfactant production by *A. aneurinilyticus* in CSL. These assays should be carried out with sterilized CSL to ensure the unique presence of *A. aneurinilyticus.* Moreover, taking into consideration the fact that *A. aneurinilytic* grows in two temperature categories, mesophilic and thermophilic, and secondary metabolites such as biosurfactants can be produced under extreme conditions of nutrients and temperature [31,32], therefore assays should include the value range for temperature between 20 °C and 60 °C. The higher value temperature is around the maximum temperature achieved during the steeping process of corn.

Therefore, the aim of this work was to explore the optimal conditions needed to produce biosurfactants in CSL, as a unique source of nutrients, in the presence of *A. aneurinilyticus* under controlled conditions to promote the production of both extracellular and cell-bound biosurfactants that could be obtained from CSL. The independent variables evaluated consisted of temperature (20–60 °C), CSL concentrations (100–400 g L^{-1}), and fermentation time (8–30 days). The dependent variables proposed in the study included the concentration of extracellular and cell-bound biosurfactants, biomass growth, critical micellar concentration (CMC), and surface tension reduction.

2. Materials and Methods

2.1. Microbial Strain and Inoculum Preparation

The strain of *A. aneurinilyticus* used in this study was isolated from CSL in previous work [33]. The lyophilized culture was labeled as CECT 9939 and maintained in the Spanish Type Culture Collection (CECT, Valencia, Spain). Inoculum preparation was conducted in an Erlenmeyer flask containing 100 mL of tryptic soy broth (TSB), which was previously sterilized at 121 °C for 15 min. The flask containing the lyophilized bacteria was incubated at 37 °C for 48 h on a rotary shaker at 150 rpm to achieve a stationary phase of growth. Then, the microorganism was reinoculated in a fresh sterilized TSB medium for 24 h under the same conditions, and this inoculum was used to start the fermentation of CSL media.

2.2. Phenotypic Characterization of A. aneurinilyticus after Fermentation

To evaluate phenotype colonies of *A. aneurinilyticus*, the inoculum was streaked on plates with tryptic soy agar (TSA) and incubated at 37 °C for 48 h. The plates with isolated colonies were observed by a Field Emission Scanning Electron Microscope (FESEM) (JSM-6700F, JEOL LTD, Tokyo, Japan).

2.3. Preparation of CSL Medium and Fermentation

For the assay, CSL provided by FeedStimulants (Zoetermeer, The Netherlands) was used. The composition of this CSL was evaluated in previous work [30]. The solutions (100 mL) of CSL (at 100, 250, 400 g L^{-1}) were prepared in 250 mL Erlenmeyer flasks by dissolving this liquor in demineralized water, which was then sterilized at 121 °C for 15 min. Then, 1 mL of *A. aneurinilyticus* was inoculated into the culture medium containing sterilized CSL solutions, and the flasks were incubated at three different temperatures (20, 40, and 60 °C) for 8, 19, and 30 days of fermentation on a rotary shaker at 150 rpm.

2.4. Experimental Design

A Box–Behnken factorial design was used to study the effect of CSL concentration, temperature, and fermentation time on biosurfactants produced by *A. aneurinilyticus*. The factors of the study were temperature (x_1) in the range between 20 and 60 °C, CSL concentration (x_2) in the range between 100 and 400 g L^{-1}, and fermentation time (x_3) in the range from 8 to 30 days, whereas the output responses were biomass (y_1), the concentration of the extracellular biosurfactant (y_2), the CMC of cell-bound biosurfactants (y_3), and the C/N ratio of cell-bound biosurfactants (y_4). Table 1 summarizes the dependent variables in the study and their variation range.

Table 1. Independent and dependent variables used in this study.

Factor	Units	Range	Code
Independent variable			
Temperature	°C	20–60	x_1
CSL concentration	g L^{-1}	100–400	x_2
Fermentation time	days	8–30	x_3
Dependent variable			
Biomass	g L^{-1}		y_1
Concentration of extracellular biosurfactant	g L^{-1}		y_2
CMC of cell-bound biosurfactant	g L^{-1}		y_3
C/N ratio of cell-bound biosurfactant	-		y_4

2.5. Quantification of Biomass

For biomass analysis, a conventional oven method was used. Samples were added to pre-dried and pre-weighed glass tubes and left at 105 °C in an oven for 48 h [34]. After drying, the final weight was determined using an analytical balance (Denver SI-234, Denver Instrument Company, Denver, CO, USA) and calculated as grams of dry weight.

2.6. Quantification of Extracellular Biosurfactants

For the quantification of extracellular biosurfactants in CSL, a calibration curve was obtained by extracting the extracellular biosurfactants produced by the bacteria in a TSB medium following the protocol established in previous work [35]. Hence, fermented TSB media were centrifuged, and the resulting supernatant was extracted with chloroform using a 2:1 (*v*/*v*) ratio (solvent: CSL solutions) at 56 °C for 60 min. Then, the organic phase was separated and vacuum distillated, resulting in the production of a biosurfactant extract, which was diluted with demineralized water to obtain a series of solutions at different concentrations below the CMC. It is well known that below the CMC, the values of surface tension in a solution were directly proportional to the biosurfactant concentration [36]. Thus, an equation relating the surface tension (ST) of the fermented CSL to the concentration of biosurfactants produced by *A. aneurinilyticus* could be obtained. Once the calibration curve was built, variations in the ST of fermented CSL in a series of dilutions were evaluated to quantify extracellular biosurfactants. For this purpose, the fermented CSL medium was centrifuged, and the ST of the supernatant was measured to know at which dilution factor the increase in the ST corresponded to the biosurfactants concentration below that of the CMC. Additionally, biosurfactants in sterilized CSL solutions (100, 250, and 400 g L^{-1}) with no inoculum addition were quantified and used as control experiments. Then, to obtain the concentration of the biosurfactant produced by *A. aneurinilyticus* under different growth conditions, the control values were subtracted from the total biosurfactants quantity measured in the inoculated CSL medium at the determined fermentation time for 100, 250, and 400 g L^{-1} CSL concentrations, respectively. The step described above was necessary because CSL, as a fermented industrial stream, already contained biosurfactants due to the spontaneous growth of microorganisms, including lactic acid bacteria and *A. aneurinilyticus* [33].

2.7. Extraction and Analysis of Cell-Bound Biosurfactants

For the extraction of cell-bound biosurfactants, the biomass was separated from the fermented medium by centrifugation at 4 °C, 4863 rcf for 15 min and was washed twice with deionized water. Then, the biomass-derived biosurfactants from each flask were subjected to phosphate-buffered saline (PBS) extraction at room temperature for 2 h, with PBS (10 mM KH_2PO_4/K_2HPO_4 and 150 mM NaCl with pH adjusted to 7.0). Subsequently, PBS containing biomass and cell-bound biosurfactants were centrifuged under the previously described conditions, and the supernatants were lyophilized using a lyophilizer Telstar LyoQuest (Terrassa, Spain) to further determine their CMC, following the methodology described by López-Prieto et al. [23]. At the end of the process, the PBS extract was centrifuged to remove the biomass and obtain the supernatant containing cell-bound biosurfactants that were lyophilized using a freeze-dryer (LyoQst InquaLab, Telstar) under vacuum conditions and then stored at 4 °C for further analysis.

2.8. Determination of ST and CMC

For CMC analysis, the collected lyophilized cell-bound extracts were re-suspended in deionized water and plunged in an ultrasonic bath (digital ultrasonic cleaner, Axtor by Lovango) for 1 min. The ST of both extracellular and cell-bound biosurfactants was measured using a Krüss K20 EasyDyne tensiometer (Krüss GmbH, Hamburg, Germany) provided with a 1.9 cm platinum Willhemly plate at room temperature. The measurements were carried out in triplicate.

2.9. Analysis of Cell-Bound Biosurfactans by Matrix Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

Cell-bound biosurfactants were subjected to Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS). The sample analysis consisted of HCCA (α-cyano-4-hydroxycinnamic acid) matrix preparation by dissolving HCCA in ethanol: 0.1% trifluoroacetic acid (TFA) with a 1:1 ratio (*v*/*v*). The biosurfactant was then

resuspended in the matrix solution with a 1:1 ratio (v/v), obtaining a final concentration of 10 mg mL^{-1}. Once the samples had been prepared, it was spotted on an MTP AnchorChip™ MALDI target (Bruker Daltonik, Bremen, Germany) and allowed to air-dry. A calibration standard (Bruker Daltonik, Bremen, Germany) was used to perform external mass calibration. Mass spectra were performed using an Autoflex III smartbeam MALDI-TOF-MS (Bruker Daltonik, Bremen, Germany) as described in a previous study [37].

2.10. Elemental Analysis of Cell-Bound Biosurfactant Extract

For the analysis of a C/N ratio in the cell-bound biosurfactant extract, elemental analysis was conducted employing an elemental analyzer (Fisons Carlo Erba EA-1108 CHNS-0, LabX, Midland, ON, Canada). The protein content could then be calculated by multiplying the nitrogen content with the nitrogen-to-protein conversion factor (6.25) [38].

2.11. Characterization of Cell-Bound Biosurfactants from CSL and TSB Medium by Fourier Transform Infrared Spectroscopy

To investigate the chemical composition, including the functional groups and chemical bonds of cell-bound biosurfactants extracted from the bacteria cultivated both in the TSB and CSL media, Fourier Transform Infrared Spectroscopy (FTIR) was employed. The biosurfactant (1 mg) was ground with 100 mg of potassium bromide and pressed with 7.500 kg for 30 s to obtain translucent pellets. Infrared absorption spectra were recorded on a Nicolet 6700 FTIR spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). The bands chosen for the analysis were in the regions 400–4000 cm^{-1}. All measurements consisted of 32 scans and a potassium bromide pellet was used as a background reference.

2.12. Statistical Treatment Data

The experimental data were analyzed by means of the response surface methodology using version 12 of Design-Expert® software (Stat-Ease, Inc., Minneapolis, MN, USA). This allowed regression coefficients to be obtained for each dependent variable in the study, which could be replaced in a generic quadratic Equation (1):

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 \quad (1)$$

where y are the dependent variables, x_i are the independent variables, β_0 is the intercept (constant), and β_i are the regression coefficients. Hence, specific quadratic theoretical equations could be established to predict the value of each dependent variable in the range established in the present study.

2.13. Production of Biosurfactant Extracts from Different Brands of CSL

With the aim of corroborating the reproducibility of CSL as a source of biosurfactants, different brands of CSL—FeedStimulants (Netherlands); Sigma Aldrich (Darmstadt, Germany); and Santa Cruz Biotechnology (Dallas, TX, USA)—were subjected to biosurfactant extraction with ethyl acetate using a 1:3 v/v CSL: ethyl acetate ratio at room temperature for 60 min, following the protocol established in previous works to obtain extracellular biosurfactant extracts from CSL [21]. Following that, comparative FTIRs were obtained in order to corroborate the similarity between the samples as described previously.

3. Results and Discussion

3.1. Evaluation of A. aneurinilyticus Capacity to Produce Biosurfactants

Before starting the examination of capacity A., *aneurinilyticus* had to produce biosurfactants in CSL under controlled growth conditions, and the biosurfactant activity of the bacteria in the TSB medium was corroborated through ST analysis. The TSB medium with no inoculation was used as a control experiment. The reduction in the initial surface tension, 56.6 mN m^{-1}, which was measured in the control, down to 37.0 mN m^{-1}, confirmed the production of extracellular biosurfactants. Moreover, the biomass obtained after the centrifugation of inoculum was subjected to PBS extraction for the detection of cell-bound

biosurfactants and their MALDI-TOF-MS analysis. Figure 1 shows the MALDI-TOF-MS spectra corresponding to the cell-bound biosurfactant extract produced by *A. aneurinilyticus* in a TSB medium. In a previous study, López-Prieto et al. [23] recorded the Electrospray Ionization Mass Spectrometry (ESI-MS) spectra for extracellular and cell-bound biosurfactants after *A. aneurinilyticus* fermentation in the TSB medium, where the peaks were observed with an m/z ratio similar to those detected in the current work for the cell-bound biosurfactant.

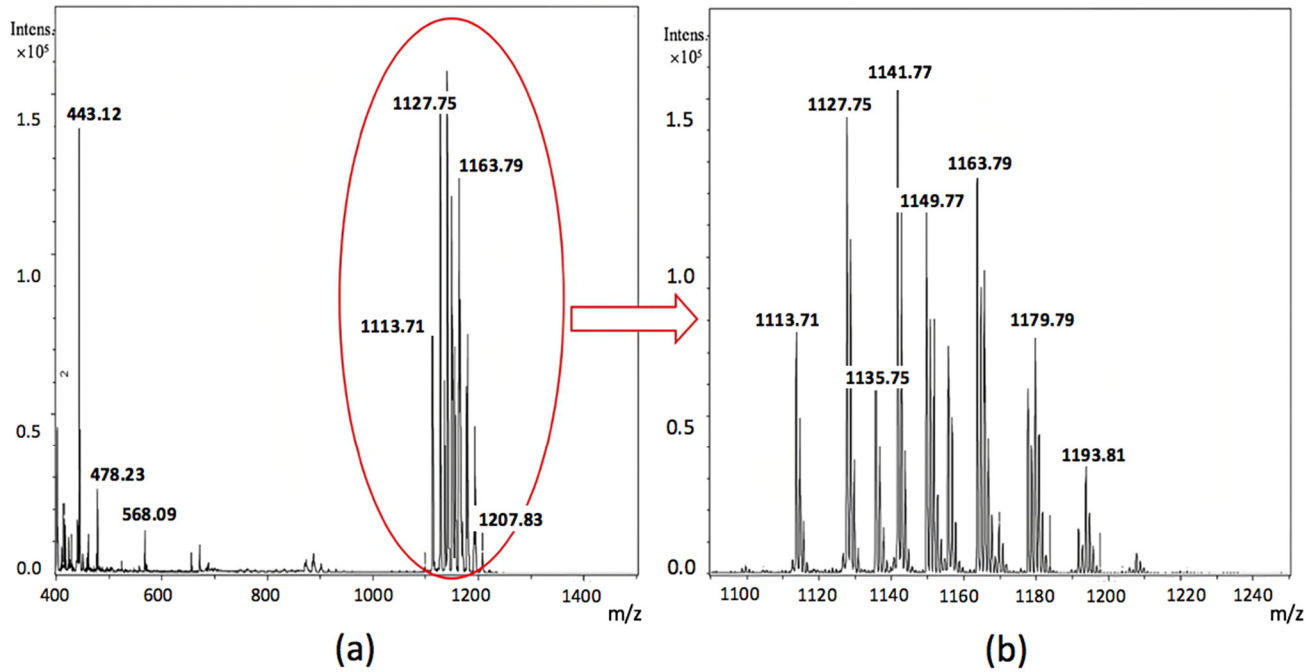


Figure 1. Mass spectra of cell-bound biosurfactants extracted from A. aneurinibacillus cells cultivated in TSB medium: (**a**) Spectrum signals in the range of 400–1500 m/z; (**b**) Spectrum zoom signals between 1090–1250 m/z.

Figure 1a shows the peak at 443 m/z that is compatible with the peak at 458 m/z in the ESI-MS spectra detected in the extracellular biosurfactant extract obtained from TSB for chloroform extraction by López-Prieto et al. [23]. Moreover, the signal at 443 m/z was also compatible with the mass at 441 m/z, as observed in previous works in the ESI-MS spectrum of the extracellular biosurfactant extract obtained from CSL by ethyl acetate extraction [39]. It can be deduced, from the spectra comparison, that the species with a 443 m/z ratio were released from the cell membrane into the extracellular phase, but it did not correspond to the lipopeptide biosurfactant. Figure 1b is an expanded segment of Figure 1a which permits a more detailed examination of the MS spectra between 1090 and 1250 m/z, where the mass spectral peak at 1141 m/z corresponds to the molecule of Gramicidin S. Moreover, in previous work [23], the cell-bound biosurfactant extract was collected after the fermentation of the TSB medium with *A. aneurinilyticus* and was analyzed by ESI-MS, where the observed peak at 571 m/z could also be attributed to the molecule of Gramicidin S. This molecule is a cyclic decapeptide with two pentapeptide moieties and the total molecular weight of 1141 Da could be visualized with a mass of 571 Da by ESI-MS, corresponding with a double charge mass. This fact does not occur when the MALDI-TOF-MS technique is applied. Additionally, *A. aneurinilyticus* produces lipopeptide biosurfactants with spectral peaks lying in the interval between 1000 and 1200 m/z (Figure 1b). It can be speculated that biosurfactants bind to the cell membrane of A. aneurinilyticus in the first stage and may then be released into the culture media, taking part in the extracellular biosurfactant extract.

3.2. CSL as a Unique Source of Nutrients in Fermentation Processes

Once the capacity of *A. aneurinilyticus* to produce extracellular and cell-bound biosurfactants had been confirmed, CSL was fermented under different environmental conditions according to the experimental design based on a quadratic model (Table 1). The quadratic model was chosen for this study because it was expected that this matched better with the production of fermentative metabolites. Linear and quadratic models do not have the same travel or range. Thus, linear models have a monotonic growth (that is, they are always increasing or decreasing), while the quadratic function increases in one section and decreases in another, which is more in agreement with the kinetic followed in the fermentative process. Several authors have applied these kinds of models that optimize fermentation conditions for biosurfactant production [40–43].

Figure 2 shows the FTIR spectra of the cell-bound biosurfactant extract of *A. aneurinilyticus* grown in TSB (control) and in CSL media under the conditions established in experiments 12 and 14.

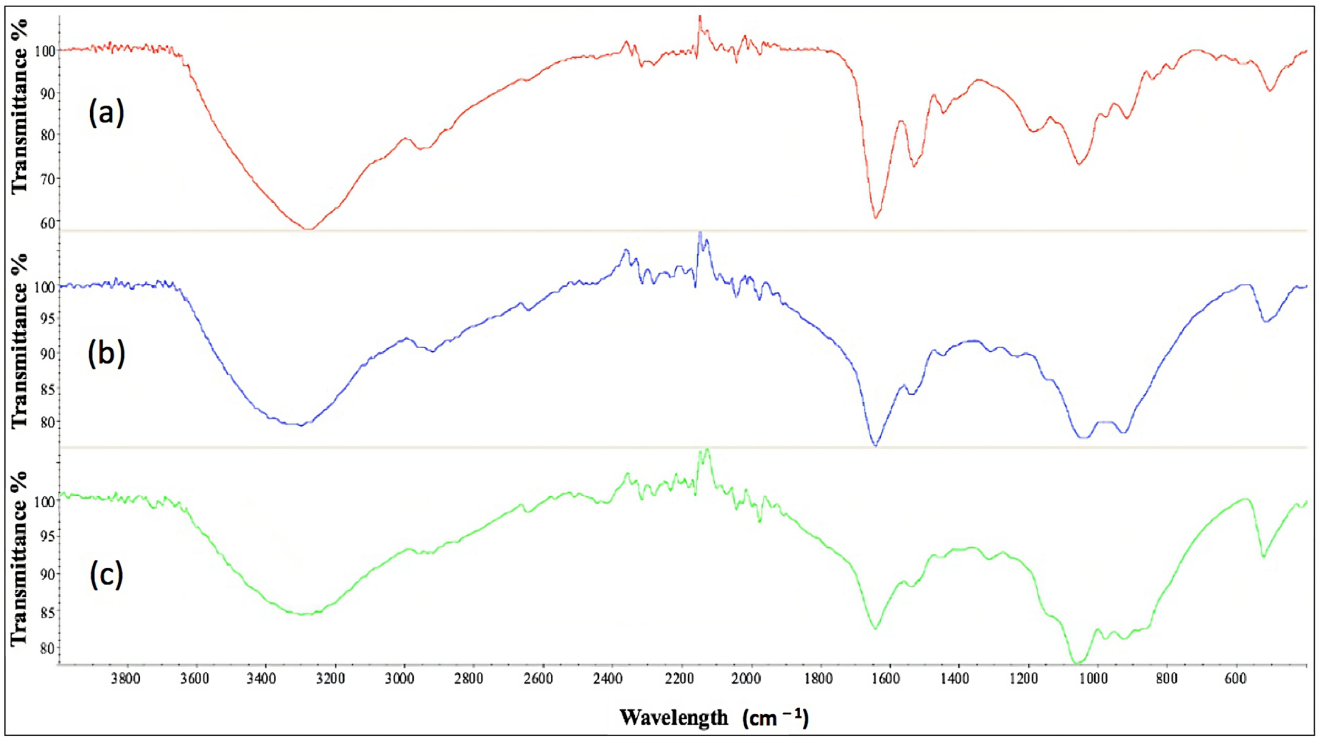


Figure 2. Comparison of FTIR spectra of the cell-bound biosurfactant of A. aneurinibacillus in TSB (**a**) In CSL media: experiment 12 and (**b**) Experiment 14 (**c**), after 48 h fermentation.

The functional groups of biosurfactants were confirmed by different characteristic peaks in all three spectra (Table 2).

Table 2. Characterization of the FTIR spectra of the cell-bound biosurfactant extracted from media.

Media	CMC (g L^{-1})	FTIR Spectrometer
TSB (a)	0.58	1071 cm^{-1}; 1537 cm^{-1}; 1634 cm^{-1}; 2934 cm^{-1}; 3275 cm^{-1}
CSL, experiment 12 (b)	1.29	1051 cm^{-1}; 1539 cm^{-1}; 1648 cm^{-1}; 2925 cm^{-1}; 3305 cm^{-1}
CSL, experiment 14 (c)	2.33	1068 cm^{-1}; 1540 cm^{-1}; 1648 cm^{-1}; 2924 cm^{-1}; 3306 cm^{-1}

Comparison of the FTIR spectra of cell-bound biosurfactants using the TSB medium (Figure 2a) and CSL solutions (Figure 2b,c) reveals some similarities for different media, with small intensity variations in the samples. The vibration signals (Table 2), at 3275, 3305, and 3306 cm^{-1}, associated with the stretching of the hydroxyl group (-OH), were

observed, where all three FTIR spectra showed a high band intensity. While the peaks at the range between 2924 and 2934 cm^{-1} likely confirmed the presence of alkyl (CH_2 and CH_3) groups of aliphatic side chains. The peaks at 1537, 1539, and 1540 cm^{-1} in FTIR spectra of TSB, CSL-12, and CSL-14, respectively, correspond to the amide functional groups from the aminoacidic chain. The next strong peaks, at 1634 and 1648 cm^{-1}, appeared in the spectrum of TSB and both spectra of CSL, respectively, due to the stretching of the carboxyl group (COO–). These vibration frequencies are similar to those obtained for biosurfactant extracts and were reported by other authors [44,45]. The vibration signals at 1071, 1051, and 1068 cm^{-1} detected in the FTIR spectra of TSB, CSL-12, and CSL-14 could be related to the presence of the ester carbonyl bond.

On the other hand, Table 3 includes the results obtained for the dependent variables, such as biomass concentration (y_1), extracellular biosurfactant concentration (y_2), the CMC of the cell-bound biosurfactant (y_3), and C/N ratio of the cell-bound biosurfactant (y_4).

Table 3. Experimental results for the dependent variables selected during controlled fermentations of CSL by *A. aneurinibacillus*.

Experiment	Independent Variable			Dependent Variable			
	x_1	x_2	x_3	y_1	y_2	y_3	y_4
1	20	250	8	1.63	2.79	1.35	5.48
2	60	250	8	3.43	4.56	1.36	5.72
3	20	250	30	1.23	0.00	1.26	5.39
4	60	250	30	4.93	3.04	1.31	5.50
5	20	100	19	0.83	0.38	2.90	5.02
6	60	100	19	1.50	1.98	2.82	6.45
7	20	400	19	4.57	0.51	2.30	5.04
8	60	400	19	7.00	1.35	2.39	6.72
9	40	100	8	0.43	0.00	1.71	5.22
10	40	100	30	0.53	2.11	3.69	5.21
11	40	400	8	4.83	1.94	1.21	4.91
12	40	400	30	6.07	2.70	1.29	4.78
13	40	250	19	1.81	6.20	2.18	7.37
14	40	250	19	1.77	5.00	2.33	7.47
15	40	250	19	2.01	6.80	2.14	6.78

Regarding the surface tension activity, extracellular biosurfactants produced by *A. aneurinilyticus* in CSL-controlled fermentation showed the capacity to reduce the ST values for the controls, at time 0, from 49.4, 47.4 and 45.5 mN m^{-1}, corresponding to 100, 250 and 400 g L^{-1} of CSL, respectively, up to 48.7, 45.9 and 43.8 mN m^{-1}. The variation in ST values in fermented CSL, when compared to the controls, was negligible because CSL already possessed biosurfactants [21]. To calculate the concentration of extracellular biosurfactants in CSL by *A.* aneurinilyticus, this was diluted several times to ensure that the biosurfactants in CSL were below the CMC; this point is reached when an increment in the surface tension is noticed after the dilution of fermented media. The maximum concentration of biosurfactants produced by *A.* aneurinilyticus in fermented CSL was between 5 and 6.8 g L^{-1}, corresponding to mild environmental conditions (40 °C, 250 g L^{-1} of CSL and 19 days) and observing maximum productivity of 0.015 g L^{-1} h^{-1}. This productivity is similar to that achieved at 20 °C, 250 g L^{-1} of CSL, and 8 days (0.014 g L^{-1} h^{-1}). However, under these conditions, only 2.79 g L^{-1} of extracellular biosurfactants were obtained. Regarding cell-bound biosurfactants, a variation in CMC values in a range between 1.2 and 3.7 g L^{-1} under different environmental conditions was shown (Table 3). The data were in consonance with the values of ST reduction and CMC measured in non-dialyzed cell-bound biosurfactants and extracted from the TSB medium fermented with *A. aneurinilyticus* [23].

After statistical treatment of the data, the theoretical equation (Equation (1)) was applied to calculate the theoretical values of the dependent variables in the value range established

for environmental conditions in the study. Table 4 includes the coefficients that should be replaced in the generic quadratic equation mentioned above for each dependent variable.

Table 4. Regression coefficients obtained after the statistical data analysis of the dependent variables in the study of CSL fermentation by *A. aneurinibacillus*.

	Dependent Variable							
	y_1	P_{y1}	y_2	P_{y2}	y_3	P_{y3}	y_4	P_{y4}
β_0	1.8633		6		2.2167		7.2067	
β_1	2.3975	<0.0001 *	0.2500	0.6402	−0.4913	0.0296 *	−0.0563	0.7731
β_2	1.0750	0.0007 *	0.9138	0.1289	0.0088	0.9593	0.4325	0.0664 **
β_3	0.3050	0.0866 **	−0.1863	0.7263	0.2400	0.2008	−0.0563	0.7731
β_{12}	0.4400	0.0820 **	−0.1750	0.8154	0.0425	0.8610	0.0625	0.8205
β_{13}	0.2850	0.2187	−0.3250	0.6668	−0.4750	0.0943 **	−0.0300	0.9131
β_{23}	0.4750	0.0661 **	0.2975	0.6931	0.0100	0.9671	−0.0325	0.9059
β_1^2	0.8858	0.0085 *	−2.9238	0.0109 *	0.5204	0.0822 **	−0.9458	0.0177 *
β_2^2	0.7258	0.0184 *	−2.0013	0.0426 *	−0.1346	0.5990	−0.4533	0.1565
β_3^2	0.2158	0.3532	−1.4013	0.1169	−0.7621	0.0246 *	−1.2308	0.0063 *

* p-values < 0.05. ** p-values < 0.1.

The coefficients with p-values < 0.05 were statistically significant with a probability higher than 95% while the coefficients with p-values < 0.1 were statistically significant with a probability higher than 90%.

He et al. [46] evaluated CSL (in the concentration range from 50 to 500 g L^{-1}) as a unique source of nutrients for the growth of *Bacillus subtilis*, which can produce lipopeptide biosurfactants, along with other metabolites. In the study [46], fermentation was carried out under specific conditions at 37 °C for 72 h, and a positive effect when increasing the cell mass in 200 g L^{-1} of CSL was shown. In the current work with *A. aneurinilyticus*, 1.2 g L^{-1} of biomass and 4.1 g L^{-1} of extracellular biosurfactants after 8 days of fermentation of 200 g L^{-1} CSL at 37 °C were predicted by the theoretical equation (Equation (1)), although after 30 days of CSL fermentation, an increase in biomass concentration up to 1.4 g L^{-1} and a decrease in the extracellular biosurfactant concentration up to 3.9 g L^{-1} was estimated. Analyzing the growth of *A. aneurinilyticus* in CSL at the highest concentration of CSL (400 g L^{-1}) at 37 °C, the highest quantity of biomass (5.6 g L^{-1}) was predicted after 30 days of fermentation, in contrast to 8 days of fermentation with predicted biomass 4.6 g L^{-1}. The data collected in the present study showed that *A. aneurinilyticus* entered a stationary phase later than *B. subtilis* did. However, as can be observed from the statistical data analysis, the period of fermentation was the least significant variable among the tested independent variables. Figure 3a shows the relationship between the bacterial biomass and two independent factors, such as CSL concentration and fermentation time, fixing the temperature at 37 °C, whereas Figure 3b shows the variation in the bacterial biomass being driven by two of the most significant variables, CSL concentration, and temperature, after 19 days of fermentation.

From Figure 3a, the maximum biomass concentration could be estimated at about 6 g L^{-1} using the highest CSL concentration (400 g L^{-1}) and the longest-running period of fermentation (30 days). It was also possible to achieve this maximum biomass concentration by reducing the fermentation time to 19 days and by increasing the temperature up to 60 °C with 400 g L^{-1} of CSL (Figure 3b).

Regarding the concentration of extracellular biosurfactants, the statistical model predicted the maximum value (6 g L^{-1}) using the intermediate values of two input variables, such as CSL concentration (250 g L^{-1}) and temperature (40 °C). Figure 4 shows the variation in extracellular biosurfactants concentration according to temperature and CSL concentration after 19 days of fermentation.

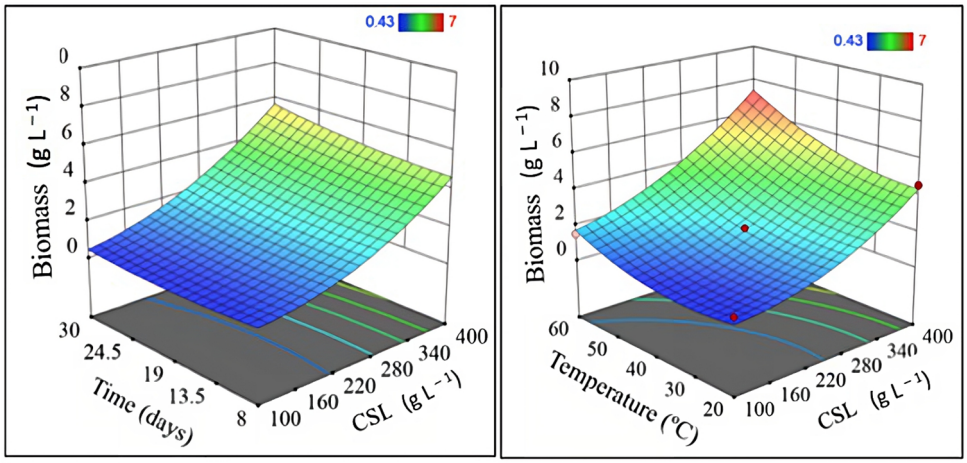


Figure 3. The 3D surface plots built by the Design- Expert® software of biomass variation in function of: (**a**) Fermentation time and CSL concentration with temperature held at 37°C; (**b**) Temperature and CSL concentration after 19 days fermentation time.

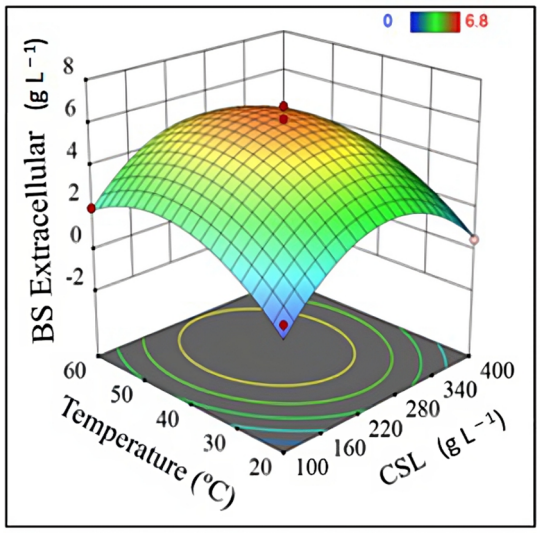


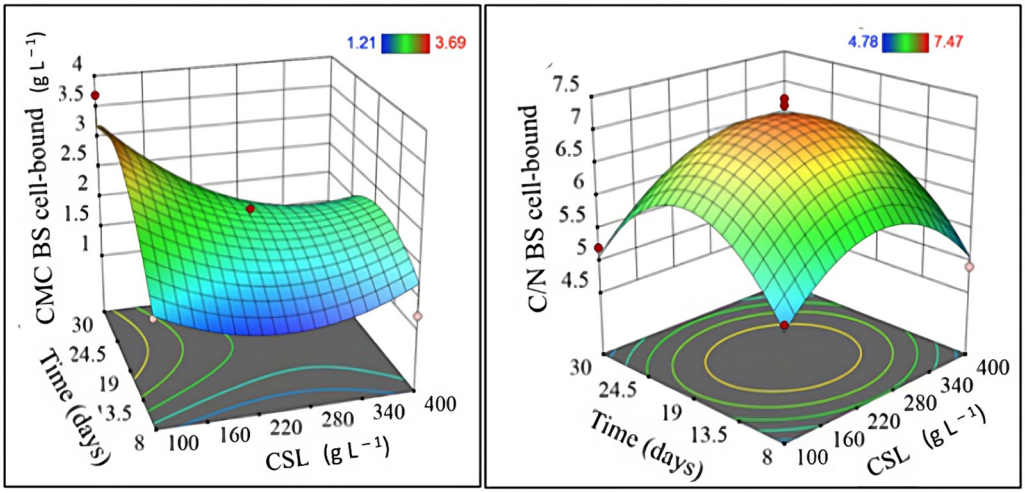
Figure 4. The 3D surface plots built by Design-Expert® software for the variation in extracellular biosurfactants concentration in the function of temperature and CSL concentration after 19 days of fermentation.

The results are in consonance with the data published by Kumar et al. [16], where *Bacillus licheniformis* produced the maximum concentration of the lipopeptide extract at 40 °C. However, this value of concentration was lower (1.9 g L^{-1}) than that obtained in the present work with *A. aneurinilyticus* using CSL as a source of nutrients. The maximum concentration of extracellular biosurfactant is within the limits of the experimental domain (Figure 4), whereas biomass increases at a higher concentration of CSL, observing that maximum biomass concentration can be outside the limits of the experimental design. However, it is important to mention that, in this case, the more relevant variables for defining the limits of the experimental design are the concentration of extracellular biosurfactants and the CMC values for cell-bound biosurfactants. This is because the aim of this work was to know those operational conditions that promote the production of extracellular and cell-bound biosurfactants. Based on this, it could be observed that optimal extracellular biosurfactant production corresponds with intermediate values of biomass that lie within the limits of the experimental domain. Moreover, it is important to mention that *A. aneurinilyticus* is a sporulated *Bacillus* strain with many variations in the phenotype, and depending

on the fermentation stage and conditions, they can produce spores that induce changes in the biomass [33]. It is also known that *A. aneurinilyticus* can produce granules linked to cells containing Gramicidin [47] that could increase the weight of the biomass, reducing the production of extracellular lipopeptides, which is consistent with the data obtained in the current work and with Figures 3 and 4.

CSL has also been evaluated as a unique culture medium for biosurfactants production using B. subtilis by Gudiña et al. [48]. In the study, the best result (1.3 g L^{-1}) was obtained using the culture medium containing 10% (*v*/*v*) of CSL, although the increment of the biosurfactant concentration up to 4.4 g L^{-1} in CSL supplemented with manganese was observed [48]. The fermentation process was carried out at specific environmental conditions, 37 °C and pH 7, to achieve a stationary phase where the biosurfactant production tended to occur.

Regarding cell-bound biosurfactant production using *A. aneurinilyticus*, at 40 °C, lower CMC values were predicted at a lower fermentation time and intermediate CSL concentrations and at higher fermentation time and at the highest CSL concentration (400 g L^{-1}) (Figure 5a).



(a) (b)

Figure 5. The 3D surface plots built by Design-Expert® software: (**a**) Variation in cell-bound biosurfactants CMC; (**b**) Variation in C/N ratio of cell-bound biosurfactants, and the function of CSL concentration and fermentation time at a fixed intermediate value of temperature (40 °C).

In this case, the temperature did not significantly influence CMC variation, contrary to the production of extracellular biosurfactants (Figure 4). Hence, Figure 5a shows the variation in the cell-bound CMC with a CSL concentration at 40 °C: the optimal temperature for extracellular biosurfactant production. Both the CMC and C/N ratio of the cell-bound biosurfactant shows a good distribution, observing optimal values within the limits of the experimental domain and, as for cell-bound biosurfactants, it is interesting to achieve lower CMC values and higher C/N ratios.

The minimum CMC value of the cell-bound biosurfactant was 1.2 g L^{-1} after 30 days of CSL fermentation (400 g L^{-1}) at 40 °C predicted by the statistical model. The calculated value of CMC after 8 days of CSL fermentation (400 g L^{-1}) at 60 °C shifted to 1.7 g L^{-1}. In general, the results obtained in the present study indicate that a longer fermentation time caused a decrease in CMC concentration and, hence, an increase in the cell-bound biosurfactant concentration within the extract when working at a high concentration of CSL; on the other hand, more favorable results of CMC were obtained at a lower fermentation time when CSL concentration was reduced at intermediate CSL concentrations. In general, a lower biomass concentration produced higher CMC values, and this was produced mainly at lower CSL concentrations. The C/N ratios were also analyzed in the cell-bound

biosurfactant extract that was obtained from *A. aneurinilyticus*. A previous work by López-Prieto et al. [23] demonstrated that *A. aneurinilyticus* possesses the capacity to produce lipopeptides and some types of antibiotics that are bound to the cell membrane. As previously discussed, the presence of these metabolites was detected in the cell-bound biosurfactant extract produced in the current study (Figure 1b). Thus, to determine the effect of the independent variables on the composition of the cell-bound biosurfactant extract, elemental analysis of the extracts was performed, and the C/N ratios of the different runs were statistically analyzed. The CSL concentration and the fermentation times were the most significant independent variables (Figure 5b). The maximum values of C/N ratios, which were consistent with a higher lipopeptide production and lower Gramicidin S production, were detected in the extract of the 250 g L^{-1} CSL solution, which was characterized by the highest concentration of extracellular biosurfactants at intermediate fermentation times. Based on these results, the simultaneous production of extracellular and cell-bound biosurfactants is possible when selecting 250 g L^{-1}, 40 °C, and intermediate fermentations times, although more favorable CMC conditions were observed at lower fermentation times, probably because further extracellular biosurfactants are still linked to the cell membrane after 8 days of fermentation. This is the first time that cell-bound biosurfactants have been explored in CSL in the presence of *A. aneurinilyticus* and observed good CMC values for cell-bound biosurfactants, taking into consideration that the extracts were not dialyzed. The process proposed was carried out at room temperature with minimal energy consumption and using phosphate-buffered saline that was both biocompatible and cost-competitive.

On the other hand, Figure 6 demonstrates experimental versus predicted data for the dependent factors using regression models.

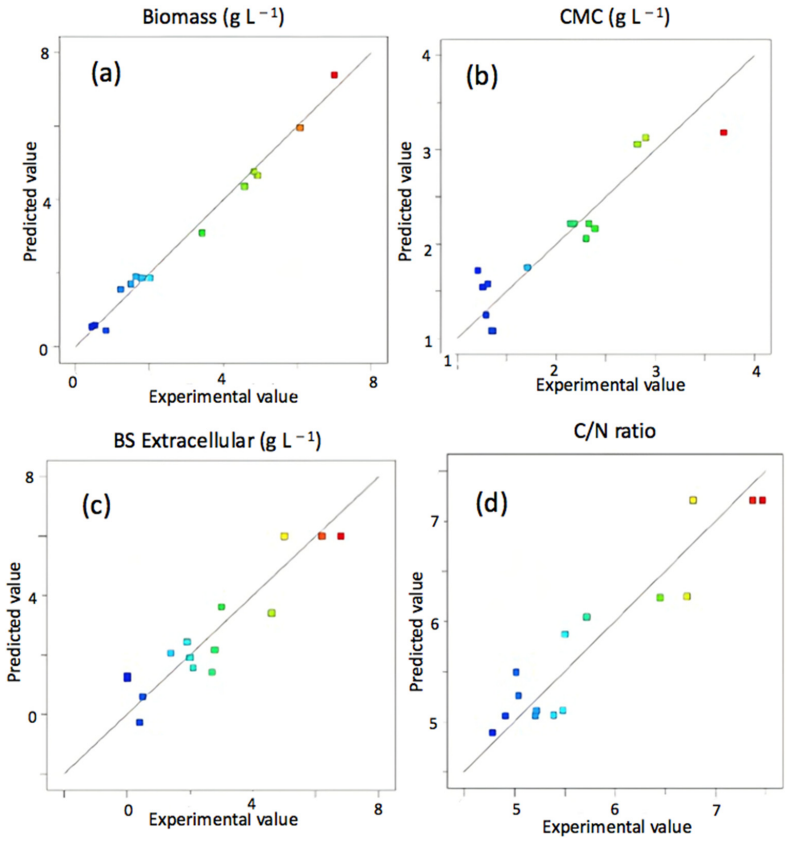


Figure 6. Plot of experimental data versus predicted values for the following dependent factors: (**a**) Biomass concentration; (**b**) CMC of cell-bound biosurfactant; (**c**) Extracellular biosurfactant concentration; (**d**) C/N ratio of cell-bound biosurfactant.

The coefficients of determination (R^2) obtained for biomass concentration, extracellular biosurfactant concentration, the CMC of cell-bound biosurfactant (Figure 6b), and the C/N ratio of cell-bound biosurfactants (Figure 6d) were 0.98, 0.85, 0.86 and 0.88, respectively.

3.3. Reproducibility of CSL as a Source for Biosurfactant Production

Figure 7 includes the FTIR comparison of different brands of extracellular biosurfactants obtained from raw CSL, observing a high similarity between the samples, between 92.2 and 98.3% in the range of 4000–400 cm^{-1} and between 96.5 and 99.6% in the range of 1800–400 cm^{-1}, which demonstrates the reproducibility of CSL as a source of extracellular biosurfactants independently of the CSL brand.

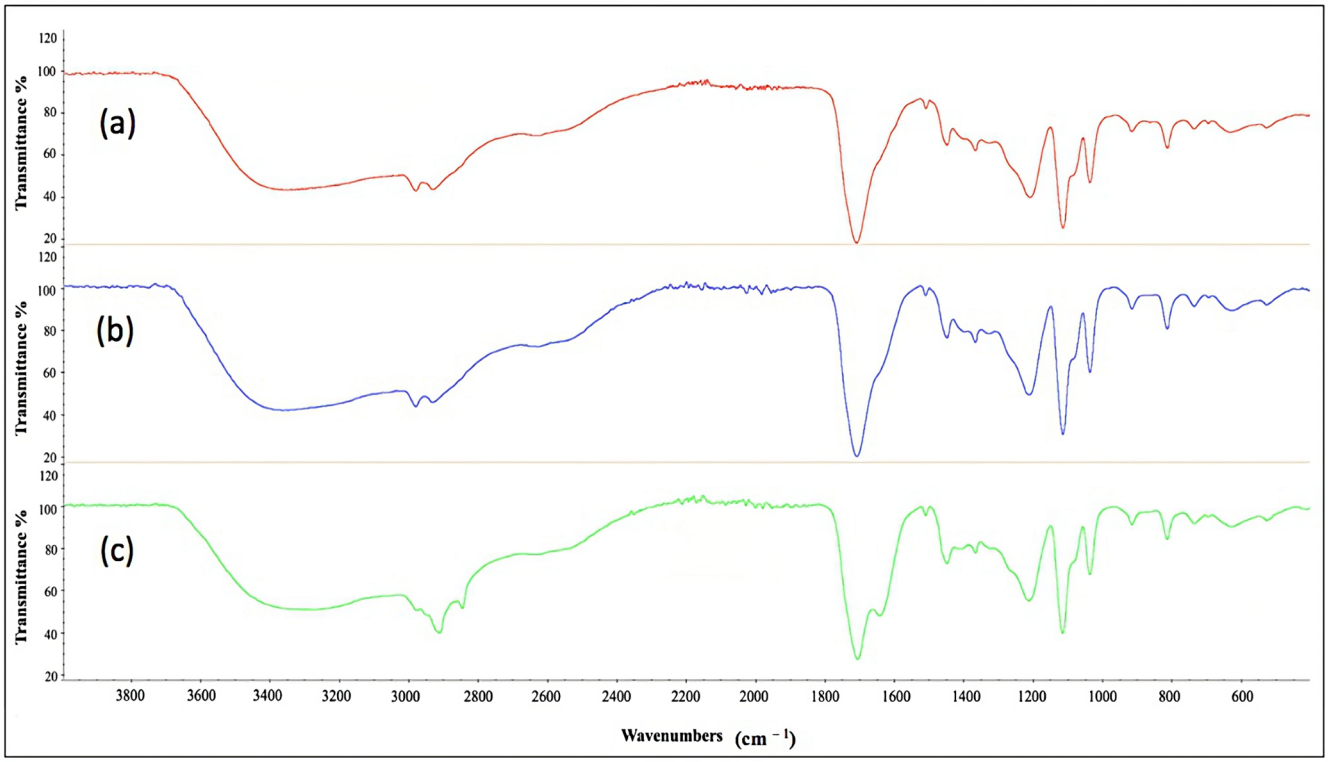


Figure 7. FTIR spectra of extracellular biosurfactant extract obtained from different brands of CSL: (**a**) FeedStimulants; (**b**) Sigma Aldrich; (**c**) Santa Cruz Biotechnology.

It is important to point out that the literature contains numerous works that propose the use of residues and secondary streams, of a different nature, as nutrients and carbon sources to produce a huge number of bioactive compounds with fermentative processes. Unlike commercial CSL, they do not contemplate quality standards [8–15]. Hence, CSL has been selected in many works, from different brands, as a supplement nitrogen source, in addition to other carbon sources, for producing bioactive compounds without checking the reproducibility between lots [28,49,50], probably because one of the commercial uses of CSL as a nutritional supplement for biotechnological processes where CSL must comply with quality standards, and that means it has an advantage over other non-commercial secondary streams.

3.4. Phenotypic Characterization of A. aneurinilyticus

The FESEM images of *A. aneurinilyticus* colonies, which were isolated from the TSB and CSL media and then streaked on TSA, were obtained, and are as shown in Figure 8.

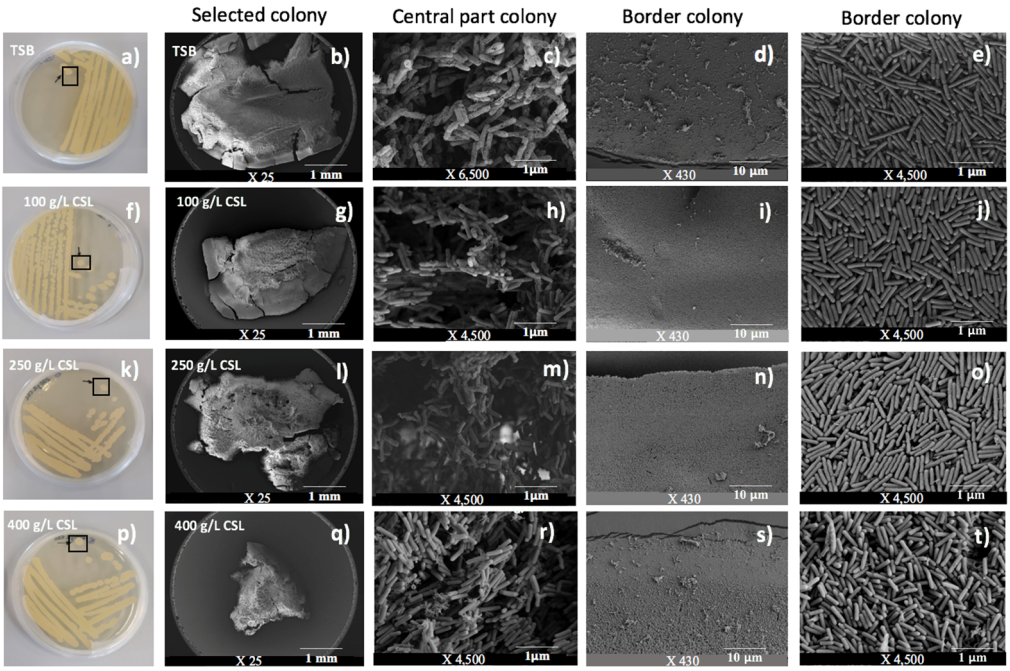


Figure 8. FESEM images of different parts of *A. aneurinilyticus* colonies, isolated from the TSB or CSL media and cultivated on TSA. (**a**–**e**) *A. aneurinilyticus* isolated from the TSB medium; (**f**–**j**) *A. aneurinilyticus* isolated from CSL solution (100 g L^{-1}); (**k**–**o**) *A. aneurinilyticus* was isolated from the CSL solution (250 g L^{-1}); (**p**–**t**): *A. aneurinilyticus* was isolated from the CSL solution (400 g L^{-1}).

A similarity in growth behavior and bacterial morphology was found between the colonies cultivated in two different media. In fact, a self-organized pattern formation could be noticed at the edges of *A. aneurinilyticus* colonies cultivated in both the TSB medium and the CSL solutions (100–250 g L^{-1}), which is a property of some biosurfactant producers such as *Bacillus subtilis* and *Bacillus meganterium* [51]. However, there was no self-organization in the center of the bacterial colony. Moreover, it was detected that the colonies of *A. aneurinilyticus* grown in the CSL solution (400 g L^{-1}) were less self-organized, which could be related to the lower production of extracellular biosurfactants detected at higher concentrations of CSL.

4. Conclusions

Regarding the production of extracellular biosurfactants, the highest concentration was detected at 40 °C using intermediate CSL concentration (250 g L^{-1}). Meanwhile, concerning cell-bound biosurfactants, lower CMC values were detected after 30 days of fermentation at the highest CSL concentration (400 g L^{-1}), which is consistent with a higher concentration in biomass (6.1 g L^{-1}) or at intermediate CSL concentrations but at reduced fermentation times (8 days). Thus, from an operational point of view, the use of 250 g L^{-1} of CSL at 40 °C and fermentation times of about 19 days was recommended to produce both cell-bound and extracellular biosurfactants as the CMC values of cell-bound biosurfactants lay under favorable values (2.1–2.3 g L^{-1}), and the concentration of extracellular biosurfactant achieved the highest concentration.

Concerning the independent variables selected in this study (temperature, CSL concentration, and fermentation time), these affected the growth of the microorganism and its capacity to produce both extracellular and cell-bond biosurfactants in different ways. The most significant independent variables for biomass growth and extracellular biosurfactant production were CSL concentration and temperature. However, the most significant input variables for CMC values regarding cell-bound biosurfactants were CSL concentration and fermentation time. From the results of this study, it can be concluded that CSL could turn out to be a very cost-competitive secondary agri-food stream for producing biosurfactants

with A. *aneurinilyticus* and under controlled fermentation using CSL as a unique source of nutrients as both extracellular and cell-bound biosurfactants could be obtained. Moreover, it is important to point out that after the extraction of extracellular and cell-bound biosurfactants, it is expected that CSL could be used as a nutritional supplement for feed or in biotechnological processes, increasing the commercial value of CSL as secondary raw material.

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