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Optimizing Carbon/Nitrogen Ratio for Biosurfactant Production by a *Bacillus subtilis* Strain

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Abstract

A *Bacillus subtilis* strain isolated from contaminated soil from a refinery has been screened for biosurfactant production in crystal sugar (sucrose) with different nitrogen sources ($NaNO_3$, $(NH_4)_2SO_4$, urea, and residual brewery yeast). The highest reduction in surface tension was achieved with a 48-h fermentation of crystal sugar and ammonium nitrate. Optimization of carbon/nitrogen ratio (3, 9, and 15) and agitation rate (50, 150, and 250 rpm) for biosurfactant production was carried out using complete factorial design and response surface analysis. The condition of C/N 3 and 250 rpm allowed the maximum increase in surface activity of biosurfactant. A suitable model has been developed, having presented great accordance experimental data. Preliminary characterization of the bioproduct suggested it to be a lipopeptide with some isomers differing from those of a commercial surfactin.

Index Entries: Biosurfactant/production; crystal sugar; lipopeptide; surface-active substances; surfactin; *Bacillus subtilis*.

Introduction

Historically, the principal driving force behind the production of surfactants has been the oil industry, which makes use of these compounds, mainly in the secondary recovery of petroleum *(1)*. However, these compounds find applications in an extremely wide variety of industrial fields involving clean-up of oil-storage tanks, transportation of heavy crude oil,

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industrial effluent treatment, ecological accident control, heavy metal removal, and bitumen recovery from tar sand, among others *(2)*.

The most commonly used surfactants come from petrochemical sources; though interest in surfactants of microbiological origin has increased considerably in recent years owing mainly to their biodegradability and low toxicity, which offer the advantage of little to no environmental impact and also allow *in situ* production *(3)*. However, the principal focus resides in the high selectivity of these compounds. Being complex organic molecules with specific functional groups gives them greater application efficiency and the capacity to perform even at extreme temperature, pH, and salinity levels *(4)*. In this context, there is a greater possibility for using surface-active microbial compounds, particularly in the areas of agriculture and medicine as well as in the pharmaceutical and textile industries *(5)*. A wide variety of microorganisms including bacteria, yeast, and molds are able to produce biosurfactants with great structural diversity *(6,7)*. However, the quantity and the chemical structure of a biosurfactant do not depend only on the microorganism that is producing it. The culture conditions, such as the carbon and nitrogen sources, trace elements, temperature, oxygen, and pH can also promote changes in the chemical structure of the molecule, and consequently, alter its physicochemical characteristics.

The lipopeptides from *Bacillus subtilis* are particularly interesting because of their strong surface activity *(8)*. Many researchers demonstrated the effectiveness of these biomolecules in reducing the surface tension to around 27 mN/m, even at concentrations as low as 0.05 g/L *(9–11)*. Moreover, these compounds present therapeutic properties, which enable them to be used in a variety of applications *(9,11)*. Some microorganisms only produce surface-active agents when cultivated in hydrocarbons. Nevertheless, biosurfactants can be produced from simple water-soluble substrates such as carbohydrates. This is of significant relevance as fermentation with carbohydrates is simpler than fermentation with hydrocarbons. The choice of the carbon source should be determined not only by its cost, availability, or nutritional characteristics of the microorganism, but also by the kind of application of the biosurfactant that is going to be produced.

Despite the advantages, biosurfactants are still economically uncompetitive because of their high production costs. The promising future of these compounds depends especially on the search for microorganisms able to produce them and on raw materials, which enable high-yields and productivity of biosurfactants with specific ends. The aim of this study was to assess the ability of a *B. subtilis* strain, isolated from a petroleum contaminated soil sample, to produce biosurfactants using low-cost raw materials in order to establish an economically feasible fermentative process. The surface-active compounds produced during fermentation were also preliminary characterized.

Materials and Methods

Microorganism

The strain *B. subtilis* YRE207 used in this study was isolated from a refinery's petroleum contaminated soil samples. The identification of the strain was done at the Coleção de Culturas do Gênero *Bacillus* e Gêneros Correlatos-CCGB, of Departamento de Bacteriologia of Instituto Oswaldo Cruz (Rio de Janeiro, Brazil), following the classic procedures based on bacteria cytomorphology, biochemistry, and physiology. The culture was maintained on BD/DIFCO nutrient agar (cod. 213000, Becton Dickinson and Company) slants at 4°C and transferred monthly.

Inoculum

The stock culture was reactivated through two successive transfers on BD/DIFCO nutrient agar slants, of 24 h each at 30°C. Then, a loopful of cells culture was inoculated on 100 mL of nutrient broth (cod. 234000), combined with 5.0 g/L glucose (Merck & Co., Brazil), and incubated at 30°C for 16 h on a rotary shaker at 150 rpm.

Biosurfactant Production

Each experiment was performed in triplicate on 500-mL Erlenmeyer flasks containing 100 mL of a mineral medium consisting of 0.1 g/L of KCl, 0.5 g/L of KH_2PO_4, 1.0 g/L of K_2HPO_4, 0.01 g/L of $CaCl_2$, and 0.5 g/L of $MgSO_4(7H_2O$ *(12,13)*, using 10.0 g/L of crystal sugar (sucrose) as the single carbon source and 4.0 g/L of NH_4NO_3, except when specified. The initial pH of the medium was adjusted to 7.0 using 1 *M* NaOH. All reagents were analytical grade (Merck). Inoculation volumes corresponding to about 0.1 g/L of exponential-phase cells were used and the flasks were incubated under the same conditions of the inoculum but this time for 48 h. Periodically, the culture purity was verified through microscopic observations using the Gram stain method.

Initially, five nitrogen sources were tested: 3.0 g/L of urea (Merck), 14.0 mL/L residual brewery yeast (Brewery Co., RJ, Brazil), 6.6 g/L of ammonium sulfate (Merck), 8.5 g/L of sodium nitrate (Merck), and 4.0 g/L of ammonium nitrate (Merck). The concentrations of the different nitrogen sources were calculated in order to obtain an initial nitrogen concentration of 1.4 g/L, a value normally used in culture media for surfactin production *(12,13)*. For the study of carbon/nitrogen ratio, the sucrose concentration was maintained constant at 10.0 g/L and the concentrations of ammonium nitrate varied: 4.0, 1.3, and 0.8 g/L, corresponding to C/N of 3, 9, and 15, respectively. In all experiments, the biosurfactant production was indirectly evaluated through the surface tension determinations in samples of cell-free fermented media.

Experimental Design

A complete factorial experimental design of two variables and three levels was used, which resulted in nine experiments. In this study, the carbon/nitrogen ratio (X_1) and agitation rate (X_2) were evaluated as the variables, according to the design matrix shown in Table 2. The variables levels were established through data obtained in the literature *(12–14)* and the percentage of the surface tension reduction adopted as the response variable for this parameter indicates the surface-active agent production.

The data were statistically analyzed using the StatSoft's Statistica 5.0 program (Statsof Inc.) through multiple regression analysis using the quadratic minimums method, taking as factors the isolated terms, the interaction, and the quadratics of the studied variables. The equation below gives the generic representation of the model:

$$Y = \beta_0 + \sum_i \beta_i X_i + \sum_i \sum_j \beta_{ij} X_i X_j + \sum_i \beta_{ii} X_i^2$$

where Y is the predicted response, β_0 is the interception coefficient, β_i, β_{ij}, and β_{ii} are, respectively, the measures of the effects of variables X_i, X_iX_j, and X_i^2. The variable X_iX_j represents the first-order interactions between X_i and X_j ($i < j$). The Statistica software was used for regression and graphical analysis of the data obtained. The results of the experiments were analyzed in order to determine the equations, the correlation coefficient R^2, the residue curve, the significant variables, the effect and the intensity of the stationary point, i.e., to set up if there was a maximum or minimum point.

Analytical Methods

Biomass

The quantifications of viable cells and spores were performed using the pour plate method. For that, samples of the fermented media were serially diluted (10^{-1} to 10^{-10}) on physiological solution (9.0 g/L NaCl) and plated on BD/DIFCO nutrient agar (cod. 213000). After 48 h of incubation at 30°C, bacteria colonies were counted using a Darkfield Quebec® colony counter (Reichter Inc.), with the results being expressed in colony forming units/mL. For spore counts, samples were heated at 80°C for 12 min before plating in order to inactivate vegetative cells. After the thermal treatment, the samples were diluted and plated as described for quantification of viable cells.

The cell concentration for inoculation of production medium was determined by dry weight according to the methodology described by Reis et al. *(13)*.

Surface Tension

Surface tensions were used as an indirect measure of surfactant production *(13)*. All measurements were made on cell-free supernatants obtained by centrifugation (13,000*g* for 20 min at 4°C). A KSV SIGMA 70

Surface Tensiometer (KSV Instruments Ltd., Finland) was used, and measurements were performed at 25°C.

Emulsification Index

The emulsifier activity was determined according to Cooper and Goldenberg *(10)*. Four milliliters of cell-free culture samples were added to six different products (gasoline, diesel oil, aviation kerosene, corn oil, soybean oil, and crude oil). After vortexing at high speed for 2 min, the mixture was allowed to settle for 24 h after which the volume occupied by the emulsion was measured. The emulsification index (E24) is the height of the emulsion layer, divided by the total height of the liquid column.

Biosurfactant Characterization

The fermented broth, previously centrifuged at 13,000*g* for 20 min at 4°C to remove the cells, was ultrafiltrated in Amicon Systems (Millipore Corporation, Brazil) through 10 kDa membranes, at pressures from 7×10^4 to 2×10^5 Pa. Then, the filtrate was analyzed by reverse phase high-performance liquid chromatography (LC10A, Shimadzu, Japan) equipped with an octadecylsilane (C-18) column (250 × 4.9 mm). The mobile phase consisted of 20% trifluoroacetic acid (3.8 m*M*) and 80% acetonitrile. Sample volume was 20.0 µL and the elution rate was 1.0 mL/min. The absorbance of the effluent was monitored at 205 nm. Surfactin from Sigma-Aldrich Co. was served as standard *(15)*.

Substrate

Sucrose levels were determined by an enzymatic colorimetric glucose-oxidase assay commercially available (Merckotest, Glucose System GOD-PAP, Merck, Germany). The basic principle is the reaction between glucose and water in the presence of oxygen and catalyzed by glucose-oxidase, which leads to gluconate and oxygenated water (H_2O_2). Subsequently, a reaction between H_2O_2, aminophenazone, and phenol occurs, producing a detectable color change. As the method is specific to glucose, the samples of the fermented broth, previously centrifuged, were hydrolyzed with a solution of 2 *M* HCl in a 1 : 1 proportion and heated at 65–67°C for 10 min. After neutralizing the samples with 1 *M* NaOH, and adequate dilution, a volume of 0.01 mL was mixed with 2.0 mL of the reagent and incubated for 30 min at room temperature (20–25°C). The absorbance of the sample was read at a wavelength of 510 nm on a DR 2500 Spectrophotometer (Hach Company, Colorado).

pH

The pH was measured using a Digimed DM-20 digital potentiometer, precision ±0.01 (Digimed Analytical, Brazil) and calibrated with pH 4.02 and 6.99 buffer solutions (Merck & Co.) at 25°C.

Table 1
Variation of Surface Tension and pH of *B. subtilis* YRE207 Fermented Media for Different Nitrogen Sources[a]

Nitrogen source	Surface tension (mN/m)[b]		Surface tension reduction (%)	Final pH
	Initial	Final		
Ammonium nitrate	66.7	31.5	52.8 ± 2.1[c]	7.0
Urea	62.4	37.3	40.1 ± 1.3	6.0
Brewery residual yeast	46.5	40.3	12.9 ± 0.4	6.5
Ammonium sulfate	64.8	40.2	37.0 ± 4.2	6.0
Sodium nitrate	61.8	54.4	11.2 ± 1	7.0

[a]Initial conditions: 10.0 g/L crystal sugar, 1.4 g/L nitrogen, 0.1 g/L cell concentration, 30°C, pH 7.0, 150 rpm, and 48 h.
[b]Mean value from triplicate measurements.
[c]Mean value ± standard deviation.

Results

Influence of the Nitrogen Source

Table 1 shows the surface tension reduction values and the pH of fermented broth for different nitrogen sources, organic and inorganic ones. A remarkable variation of the surface tension reduction percentage regarding the nitrogen source used was observed, although the final pH has not changed considerably. Among the nitrogen sources tested, ammonium nitrate, ammonium sulfate, and urea were the most favored ones for the biosurfactant production. Nevertheless, ammonium nitrate was the best nitrogen source for surface-active compound synthesis, because the lowest surface tension value and its higher percent reduction were achieved under this nutritional condition. In the final stage of growth, the cells cultivated in the presence of sodium nitrate showed a distinct morphology when compared with that grown in the other tested conditions. Clusters formation could be seen on the fermented medium, which could have favored the cell separation from the medium at the end of the process.

Effect of the Carbon/Nitrogen Ratio and the Agitation Rate on the Biosurfactant Production

The surface tension values determined for the different agitation rates and C/N ratios, using sucrose and ammonium nitrate, and after 48 h, are shown in Figs. 1–3. For C/N 3, the increase in the agitation from 50 to 150 rpm promoted only a slight increase in the total viable cell concentration; however, a remarkable decrease of cellular population at 250 rpm was observed (Fig. 1). Sporulation presented distinct variation, and the highest number of spores were observed under the higher agitation rate condition. Perhaps the increase in the agitation rate caused a greater mass transfer,

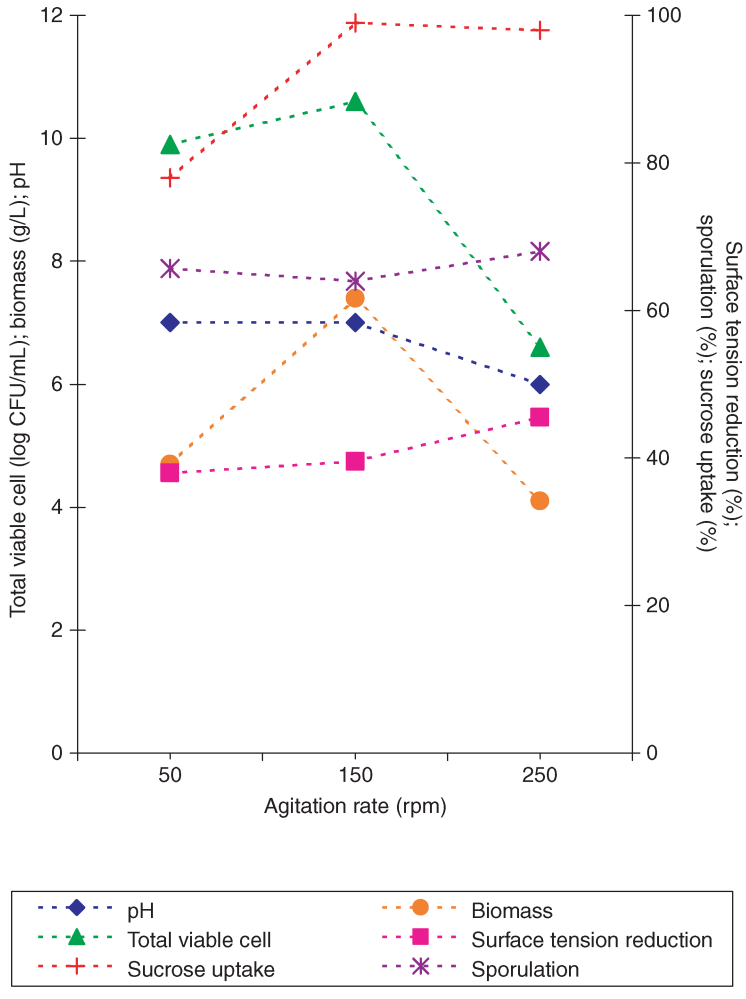

Fig. 1. Results for *B. subtilis* YRE207 fermented medium using C/N ratio of 3 at different agitation rates.

which led to faster sugar consumption; sporulation and cell death were a consequence of nutritional depletion.

The reduction of the surface tension was analogous to the percentage of sporulation, suggesting that the synthesis of the surface-active compound was stimulated at the end of the exponential phase of growth with the spore formation. Agitation promotes nutrient and oxygen transfers in the culture media, which in turn favors microbial activity. So, usually, a faster growth of aerobic microorganisms is achieved with high-agitation rates. The oxygen availability favors the biosynthesis reactions, as they are energy-dependent, resulting in a higher quantity of produced biomass, and consequently, in a higher probability of spores formation. However, it is difficult to reach the maximum growth in agitated flasks because in this condition, the oxygen supply is limited. Additionally, in the exponential

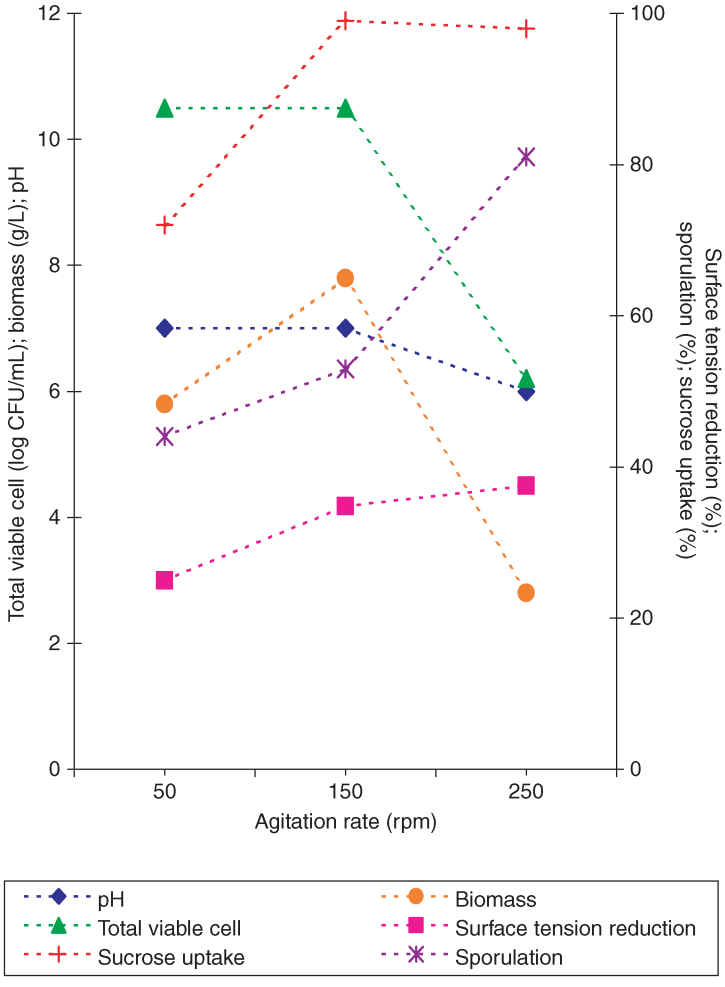

Fig. 2. Results for *B. subtilis* YRE207 fermented medium using C/N ratio of 9 at different agitation rates.

phase of growth, the intense propagation of cells causes depletion of oxygen faster than its dissolution on the medium.

A 78% substrate uptake was obtained in the experiment conducted with an agitation rate of 50 rpm. Higher uptakes, of about 99%, were evidenced for the experiments under 150 and 250 rpm. A decrease of the pH was detected when the process was conducted under 250 rpm. A similar behavior was verified for the experiments with C/N ratio of 9 (Fig. 2). Under this condition, a remarkable increase in spore formation directly related to the agitation rate increase was observed. On the other hand, except for the substrate uptake, the bacterial strain showed a different performance when it was cultivated in the media with C/N of 15. In this condition, the increase in the agitation rate promoted an augmentation of the dry weight. However, a reduction in the percentage of sporulation and

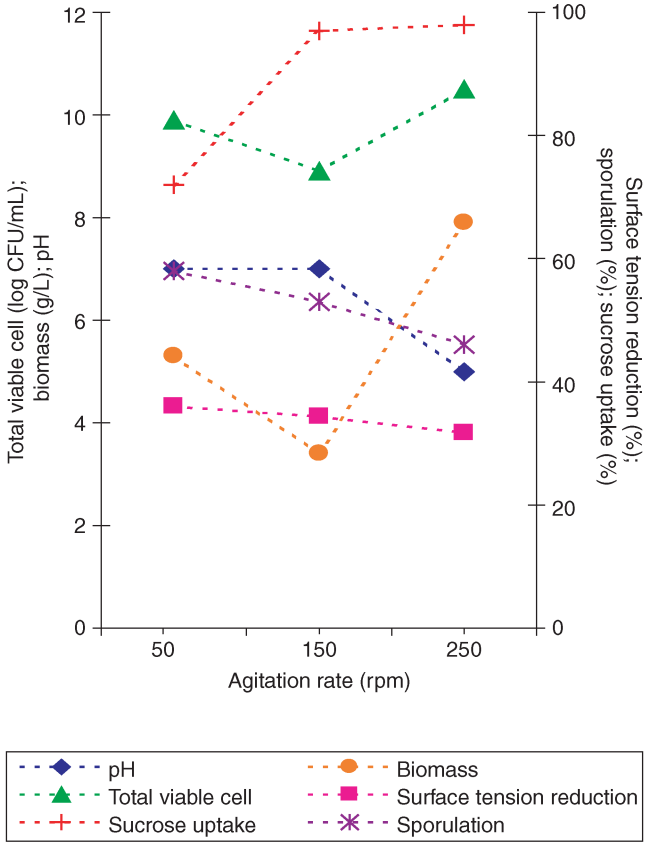

Fig. 3. Results for *B. subtilis* YRE207 fermented medium using C/N ratio of 15 at different agitation rates.

surface tension reduction occurred. At the end of the process, samples of the fermented broth were evaluated to its emulsifying activity. All the samples were able to emulsify completely light Arab oil. Nevertheless, no stable emulsions were detected for aviation kerosene, gasoline, diesel oil, and corn and soybean oils. Therefore, the biosurfactant produced may be applied in enhanced oil recovery, bioremediation of oil, and oil tank clean up, but not, as it appears, for use in the food industry.

Statistical Analysis

The optimization of the carbon/nitrogen ratio (X_1) and agitation rate (X_2) variables was performed considering the percentage of surface tension reduction. From the results obtained, the multiple regressions were done taking as the factors the isolated terms, the interactions, and the quadratics of the variables studied. Table 2 shows the surface tension values for the media before and after fermentation for each experiment.

After the multiple regressions using the Statistica 5.0 program, the parameters with a significance level superior to 10% of the Student's *t*-test

Table 2
Surface Tension Values in Different Experimental Conditions

Experiment	X_1 (C/N)	X_2 (agitation rate [rpm])	Surface tension (mN/m)[a]		Surface tension reduction (%)
			Initial	Final	
1	3 (–)	50 (–)	66	40.7	38.3 ± 0.4[b]
2	3 (–)	150 (0)	66	39.9	39.5 ± 1.3
3	3 (–)	250 (+)	66	35.7	45.9 ± 0.6
4	9 (0)	50 (–)	57.7	40.8	29.3 ± 1.2
5	9 (0)	150 (0)	57.7	37.6	34.8 ± 0.7
6	9 (0)	250 (+)	57.7	36.8	36.2 ± 1.1
7	15 (+)	50 (–)	60.7	38.4	36.7 ± 0.1
8	15 (+)	150 (0)	60.7	39.4	35.1 ± 0.9
9	15 (+)	250 (+)	60.7	41	32.5 ± 0.3

[a]Mean value from triplicate measurements.
[b]Mean value ± standard deviation.

were eliminated. So, the isolated terms and the agitation quadratic (X_2, X_2^2) were dismissed. The C/N ratio isolated was the only significant variable on the percentage of surface tension reduction. The correlation coefficient (R^2) of 0.803 indicates an adequate adjustment of the experimental data on the percentage of surface tension reduction response, showing that 80.3% of the data variability was explained by the empiric equation that was proposed. The residue distribution around zero and the representation of expected values vs the observed values are shown in Figs. 4 and 5. The residues distribution was at random around zero, presenting no tendency about distribution (Fig. 4). Figure 5 shows that the experimental responses for the percentage of surface tension reduction were near the values given by the empiric equation. Eliminating the insignificant parameters we obtain the following equation:

$$\text{Surface tension reduction (\%)} = 33.4333 - 3.2333\,X_1 + 4.5666\,X_1^2 - 2.95\,X_1X_2$$

This equation represents the adjusted model for the prediction of the surface tension reduction with X_1 and X_2 in the codified form. The variables were determined through the following codifying equations: carbon/nitrogen ratio (X_1) = [C/N – 9]/[(15 – 3)/2] and agitation rate (X_2) = [AG (rpm) – 150 (rpm)]/[(250 (50)/2] (rpm).

From the complete equation (surface tension reduction (%) = 33.4222 – $3.2333X_1 + 1.7166X_2 + 4.5666X_1^2 + 0.0166X_2^2 - 2.951X_1X_2$), an algorithm of the *Maple V release 4* (Canada) program was used to calculate the stationary point. The calculation of this point gave the following values: $X_1 = 0.590186$ and $X_2 = 0.731199$. The coordinates of the stationary point are between the experimental regions. The λ values that refer to the percentage of the

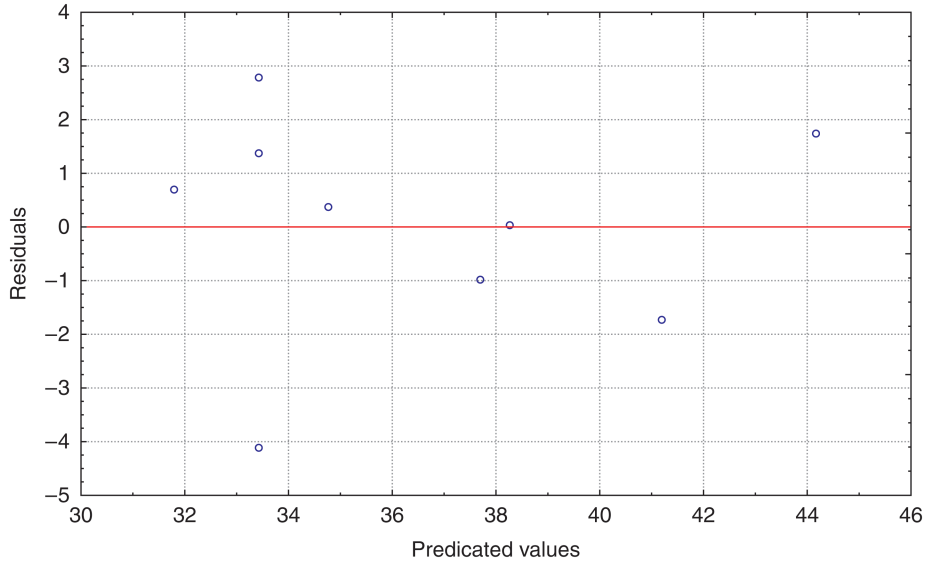

Fig. 4. Relative residues distribution in relation to the surface tension reduction.

surface tension reduction indicated that this response has a saddle point because λ_1 (–0.419649) and λ_2 (5.002989) have different signs. Using the same algorithm of the *Maple V release 4* program, the codified variable values (*X*'s) were calculated that correspond to the maximization of the response, yielding the following values $X_1 = -1$ and $X_2 = 0.98$. Using the codification equations, the real values may be determined for the variables' concentration in the maximization point of surface tension reduction: $X_1 = 3$ for the C/N ratio and $X_2 = 248$ rpm for the agitation. The determination of the stationary point through canonical analyses using the complete model, in this case, shows that the model really represents the behavior of the surface tension reduction as a function of the studied variables.

Figure 6 illustrates the effect of the studied variables on the surface tension reduction. It is evident that for the lowest values of the C/N ratio, higher rates of agitation cause higher percentage of surface tension reduction. On the other hand, the condition of maximum point of the percentage of surface tension reduction occurs for lower values of the C/N ratio and for higher values of agitation rate. This behavior was also observed in the analyses of the experimental results.

Characterization of the Biosurfactant Produced

Preliminary experiments to determine the presence of surfactin in the fermented broth were performed through chromatographic analyses. Figure 7 shows the chromatograms for the commercial surfactin (Sigma) and for the biosurfactant produced under C/N of 3 and 250 rpm conditions.

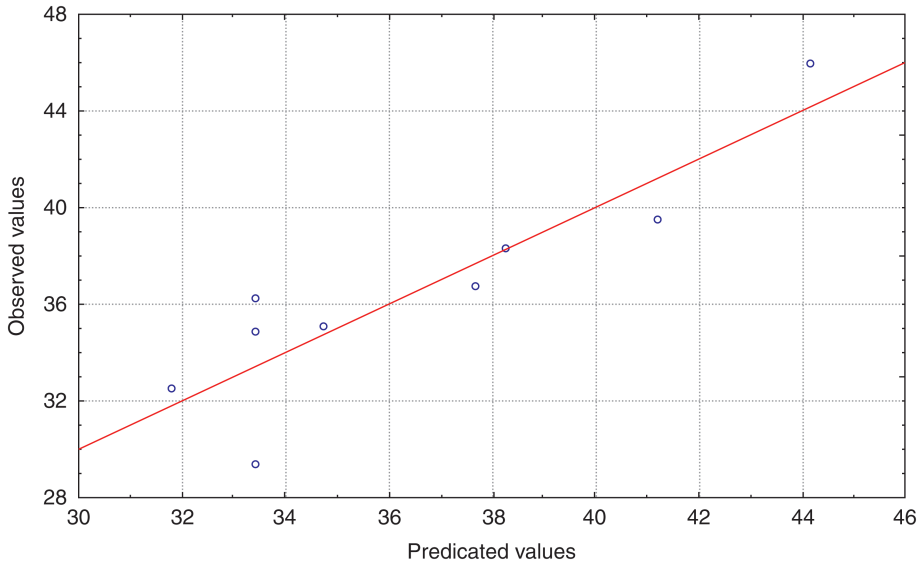

Fig. 5. Correlation between experimental values and values predicted by mathematical model.

Surfactin from Sigma presented nine retention peaks. Comparatively, the biosurfactant produced in this study showed similarity with the standard (Sigma) in four retention peaks (number of the peak/retention time: 4/18.593; 5/20.316; 7/25.207; and 8/25.753). Wei and Chu *(15)* observed a chromatographic profile for the surfactin obtained from *B. subtilis* ATCC 21332 similar to the standard (Sigma) also showing nine peaks of which eight were at the same retention time.

Discussion

Among the nitrogen sources studied, besides the addition of the residual brewery yeast, the addition of sodium nitrate or the ammonium sulfate was not able to significantly reduce the surface tension of the fermented medium (Table 1). According to Davis, Linch, and Varley *(12)*, the type and the concentration of the nitrogen source are important for the optimization of secondary metabolite production, which most of the biosurfactants are. However, the number of publications in the literature about the qualitative and/or quantitative influence of the nitrogen source on the biosurfactant production by *B. subtilis* is still low. Moreover, as stated previously, the production of surfactin by *B. subtilis* in sucrose medium is growth-associated *(13)*.

The production medium containing urea or ammonium nitrate as the nitrogen source was the best in stimulating the biosurfactant production by *B. subtilis* YRE 207, resulting in percentages of surface tension reduction of 40 and 53%, respectively (Table 1). According to Ramnani et al. *(16)*, *Bacillus* spp. are able to reduce the surface tension around 30–60%. Therefore, both

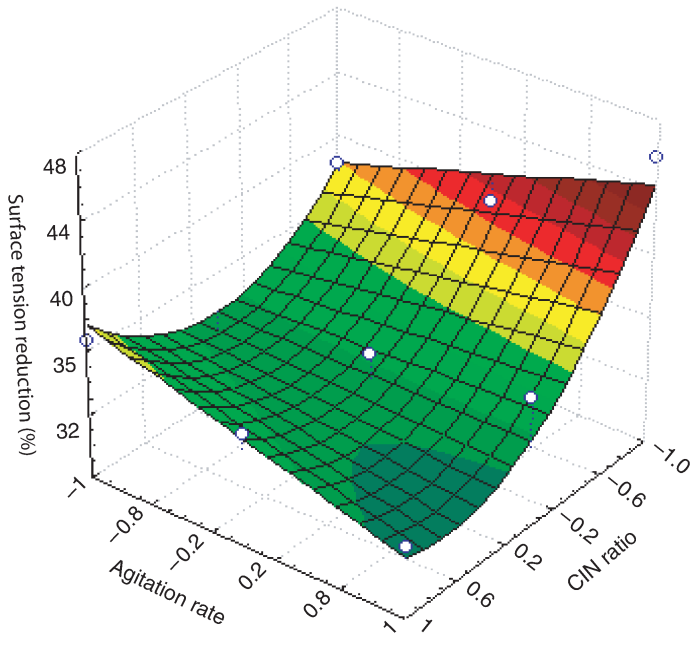

Fig. 6. Effect of C/N ratio and agitation rate on surface reduction tension.

nitrogen sources studied were adequate, making the ammonium nitrate the most favorable to the biosurfactant synthesis for the bacterial strain used.

Makkar and Cameotra *(17)* obtained the maximum lipopeptide production by a *B. subtilis* strain after 72 h of cultivation at 45°C with urea and nitrate ion on 3 g/L concentrations. Similarly, the cultivation of *Pseudomonas aeruginosa* strain in different nitrogen sources ($NaNO_3$, NH_4NO_3, $(NH_4)_2SO_4$, NH_4Cl), with concentrations varying from 2.0 to 5.0 g/L, presented satisfactory surface tension reduction of the medium in the presence of 2.0–3.0 g/L of $NaNO_3$ *(18)*.

Davis, Lynch, and Varley *(12)* concluded that when ammonium ions are used as a nitrogen source, *B. subtilis* ATCC 21332 growth is favored. Meanwhile, the nitrate ion is consumed during *B. subtilis* secondary metabolism, i.e., when the biosurfactant is actually being produced. That is why the evaluation of the nutrients sources is so important. In this work, the effect both of the carbon/nitrogen ratio and the agitation rate were evaluated through an experimental design (Table 2). Variations in the C/N ratio (X_1) and in the agitation rate (X_2) resulted in alterations of the surface tension reduction (Table 2) varying from 29.3 to 45.9%. Comparing these results, it is possible to establish that in the lower C/N ratio (3 and 9), the increase in the agitation rate promoted an increase in the percentage of surface tension reduction. However, for C/N 15 condition, an increase in the agitation rate caused a small reduction in this percentage. The best results were obtained for the medium containing crystal sugar (10.0 g/L) and NH_4NO_3 (4.0 and 1.3 g/L) under 250 rpm agitation rate, corresponding to C/N of 3 and 9.

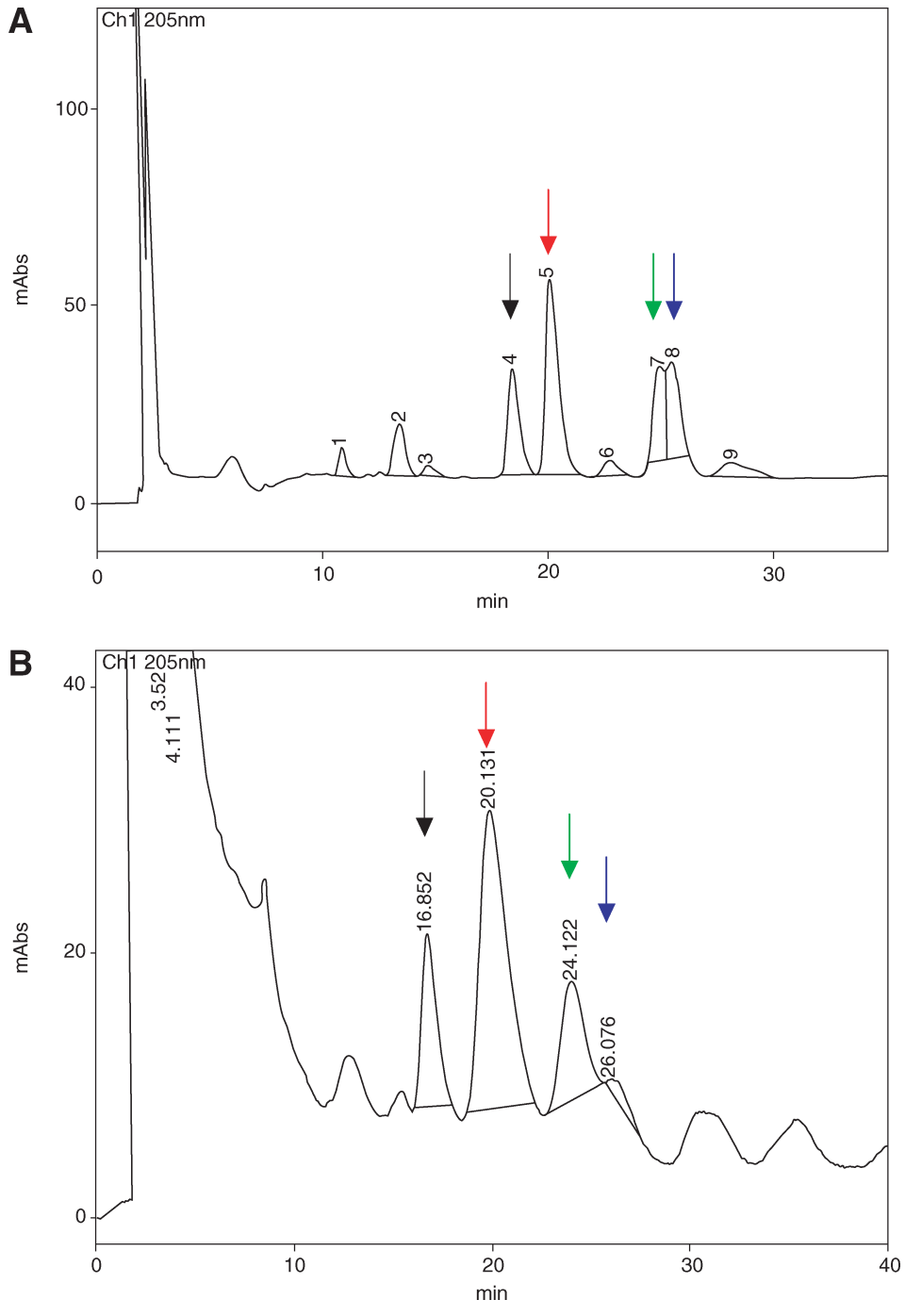

Fig. 7. Chromatographic profile of the surfactin **(A)** Sigma and **(B)** fermented broth for C/N ratio of 6 and 250 rpm (peaks and correspondent retention times for Sigma surfactin: 4/18.593; 5/20.316; 7/25.207; and 8/25.753).

The literature usually adopts a carbon/nitrogen ratio around three and agitation of 150 rpm. Davis, Lynch, and Varley *(12)* obtained the highest quantity of surfactin (439.0 mg/L) cultivating *B. subtilis* ATCC 21332 in a medium-containing glucose (10.0 g/L) and ammonium nitrate (4.0 g/L), which corresponds to a C/N ratio of 11. In the experiments performed under 150 and 250 rpm agitation rate, for the different combinations of C/N, an almost total sucrose uptake from the 48 h fermented media was evidenced. Nitschke and Pastore *(11)* showed similar results when using cassava waste water. The pH values obtained at the end of the fermentation were between neutral and acid, 5.5 being the lowest value obtained (Figs. 2 and 3). Wei and Chu *(15)* also observed the existence of an acidogenic metabolic pathway for a high concentration of iron. According to Claus and Berkeley *(19)*, the ideal pH for *B. subtilis* growth is between 5.5 and 8.5. Makkar and Cameotra *(17)* observed remarkable decreases in the growth and mainly in the quantity of biosurfactant produced by a strain of *B. subtilis* when the medium pH was adjusted to 4.5. Additionally, the stability of the surfactin molecule was studied in different pH values *(11)*. The reduction of the pH to values lower than 5.0 favored the microbial surfactant precipitation, which resulted in an elevation of the medium surface tension. So, it may be concluded that there was no influence of the pH on the metabolic activity of the bacterial strain studied.

Analyzing the results obtained, it can be affirmed that there was no correlation between growth and surface tension variation (Figs. 1–3). However, under the conditions in which a higher sporulation was verified (C/N 3—250 rpm; C/N 9—250 rpm, and C/N 15—50 rpm), a decrease in the surface tension was also noted. Branda et al. *(20)* suggest that the surfactin production is related to the sporulation process in *B. subtilis*. The chromatograms of the recovered and partially purified surfactin (C/N 3 and 250 rpm) and of a sample of commercial surfactin (Sigma) showed peaks at different retention times, which prove the existence of different isomers. On the other hand, four peaks were similar to the preponderant peaks of the standard surfactin. Therefore, there is a possibility that the biosurfactant produced in this work has different physicochemical properties that could make it suitable for other types of applications. Obviously, further investigations will be needed.

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