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Study of the Synergistic Effects of Salinity, pH, and Temperature on the Surface-Active Properties of Biosurfactants Produced by *Lactobacillus pentosus*

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ABSTRACT: Many studies have investigated the effects of pH, temperature, and salinity on the surface-active properties of various surfactants, although in most cases the variables have been studied separately, without considering the effects of any interactions between them. In the present study, a Box−Behnken factorial design was applied to study the effects of pH, temperature, and salinity on the surface-active properties of a biosurfactant produced by *Lactobacillus pentosus*. The data obtained enabled development of a second-order model describing the interrelationships between operational and experimental variables, by equations including linear, interaction, and quadratic terms. The variable that had the greatest effect on the surface-active properties of the biosurfactant was pH. Moreover, at pH 3−5.5, decreases in salinity and temperature acted synergistically, reducing the surface tension of the biosurfactant; at pH 8, the same effect was observed with increasing salinity and temperature.

KEYWORDS: *surface tension, emulsion volume, emulsion stability, surface response methodology*

INTRODUCTION

Surface-active agents, known as surfactants, are present in many formulations of commercial products such as detergents, motor oils, pharmaceuticals, and agricultural products.[1] Surfactants, of chemical or biological origin, are increasingly used in the bioremediation of aquatic ecosystems and soils, since they facilitate the solubilization of contaminants, increasing their bioavailability and therefore their subsequent elimination via the action of microorganisms.[2−7]

Some studies have investigated the use of surfactants of chemical origin for the bioremediation of contaminated matrixes,[8,9] as these are usually cheaper and are more readily available to the consumer than biosurfactants, which are synthesized by living cells and obtained by biotechnological methods. However, there are many advantages associated with the use of the latter. Biosurfactants have surface−activity properties that make them excellent emulsifiers, foaming, and dispersing agents. They are environmentally friendly, biodegradable, less toxic than chemical surfactants, and nonhazardous. They are usually active at extreme temperatures, pH values, and salinities and can be produced from industrial waste and byproducts.[9,10] For instance, vine pruning waste, grape marc, and other lignocellulosic residues such as hazel nut and walnut shells can be used as substrates for *Lactobacillus pentosus* to produce biosurfactants.[9,10] This makes cheap production of biosurfactants possible and allows waste substrates to be utilized, thus reducing their polluting effect. In a previous study,[10] it was demonstrated that biosurfactants produced by *L. pentosus* are stable at extreme temperatures and are more active at high pH than at low pH, although neither the synergistic effect of these variables or the effect of salinity on the surface properties of this biosurfactant have been tested.

The effect of salinity on the surface tension of biosurfactants is very important, as bioremediation of aquatic ecosystem may occur at a wide range of salinities. In a study of the effects of mixtures of *Enterobacter cloacae* and *Pseudomonas* sp. (ERCPPI-2) on biosurfactant production, Darvishi et al.[11] found that biosurfactant production occurred at a salinity of up to 15%, although optimal production occurred on a minimal salt medium (1%). Other authors[12−14] did not observe any degradation or decrease in the biosurfactant activity at different salinities. However, Abouseoud et al.[15,16] reported that salinity affected the emulsifying capacity and solubilization of naphthalene of a biosurfactant produced by *Pseudomonas fluorescens*. Mnif et al.[17] also reported increased biosurfactant stability with increasing salinity.

In the present study, the synergistic effects of salinity, pH, and temperature on the surface properties of biosurfactants produced by *L. pentosus* were investigated by applying an incomplete factorial design. Moreover, the emulsifying capacity of the biosurfactant for stabilized gasoline/water emulsions was evaluated under different conditions of salinity, pH, and temperature.

MATERIALS AND METHODS

Hydrolysis of Vine Pruning Waste. Ground samples of vine pruning waste were hydrolyzed under selected conditions (3% H_2SO_4, 15 min, 130 °C, and liquid/solid ratio 8:1 g/g), and the liquid obtained was neutralized with $CaCO_3$ to a final pH of 6.5. The precipitated $CaSO_4$ was separated from the supernatant by filtration.

Microorganisms. *L. pentosus* CECT-4023 T (ATCC-8041) was obtained from the Spanish Collection of Type Cultures (Valencia, Spain). The strain was grown on MRS agar. Inocula were prepared by solubilization of cells from plates with 5 mL of sterilized hydrolysate.

Fermentation of Hemicellulosic Sugars from Vine Pruning Waste by *L. pentosus*. The clarified hydrolysates were supplemented with nutrients (10 g/L of yeast extract and 10 g/L of corn steep liquid), sterilized, and used directly as fermentation media. The chemostat

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fermentation was carried out in a 2 L Applikon fermentor at 200 rpm with a working volume of 1.6 L at 31 °C, and the pH was adjusted to 5.85 for 48 h. Once the fermentation was complete, the *L. pentosus* biomass was separated from the fermentation medium by centrifugation to extract the biosurfactant.

Extraction of Biosurfactants. Cells were recovered by centrifugation, washed twice in deionized water, and resuspended in 50 mL of phosphate-buffered saline [PBS: 10 mM KH_2PO_4/K_2HPO_4 and 150 mM NaCl (pH adjusted to 7.4)], following the protocol described by Portilla-Rivera et al.[10] The fermentation media containing biomass/liquid (PBS) relationship used during the extraction of biosurfactants was 6:1. The bacterial suspensions were kept at room temperature for up to 2 h with gentle stirring to encourage release of the biosurfactant. The bacteria were then removed by centrifugation, and the supernatant liquid containing the biosurfactants was tested for surface activity. The concentration of biosurfactant utilized in this work was the CMC, and the solution contains about 2.2 mg/L of surfactin equivalents in PBS extract.

Experimental Design: The Box–Behnken Response Surface Methodology. The response surface methodology consists of a group of mathematical and statistical techniques based on fitting empirical models to the experimental data obtained in relation to experimental design.[18] Box–Behnken designs are a class of rotatable or nearly rotatable second-order designs based on three-level, incomplete factorial designs.[19] The number of experiments (N) required for a full Box–Behnken design are given by the formula $N = 2k(k - 1) + C_0$, where k is the number of factors and C_0 is the number of central points.[20] The simplest equation describing a linear function is described by eq 1.

$$y = \beta_0 \sum_{i=1}^{k} \beta_i x_i + \varepsilon \quad (1)$$

where β_0 is the constant factor, β_i represents the coefficients of the linear parameters, k is the number of variables, x_i represents the variables, and ε is the residual factor associated with the experiments. When the experimental data do not fit a linear equation, then it is desirable to include levels in the input variables. In this case, a polynomial response surface must be generated. Box–Behnken experimental designs were constructed for situations in which it is desirable to fit a second-order model (eq 2).

$$y = \beta_0 \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \sum_{j \geq i}^{k} \beta_{ij} x_i x_j + \varepsilon \quad (2)$$

where β_{ij} represents the coefficients of the interaction parameters. These designs include a central point used to determine the curvature, and critical or optimal conditions are deduced from the above second-order function by including quadratic terms (eq 3).

$$y = \beta_0 \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1}^{k} \sum_{j \geq i}^{k} \beta_{ij} x_i x_j + \varepsilon \quad (3)$$

where β_{ii} represents the coefficients of the quadratic parameters. Thus, the experimental data enable the development of empirical models that describe the interrelationship between operational and experimental variables by equations including linear, interaction, and quadratic terms.

The range of independent and dependent variables studied is included in Tables 1 and 2, respectively. The standardized (coded) dimensionless independent variables used, with variation limits (−1, 1), were defined as x_1 (salinity), x_2 (pH), and x_3 (temperature).

Thus, the quadratic function obtained for all three variables is described in eq 4.

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 + \beta_{11} {x_1}^2 + \beta_{22} {x_2}^2 + \beta_{33} {x_3}^2 \quad (4)$$

where y is the dependent variable, β denotes the regression coefficients (calculated from experimental data by multiple regressions using the least-squares method), and x denotes the independent variables.

Table 1. Independent Variables Used in the Study

Independent Variables			
variable	nomenclature	units	range of variation
salinity	[NaCl]	%	1–5
pH	[pH]		3–8
temperature	[T]	°C	4–56
Dimensionless, Coded Independent Variables			
variable	nomenclature	definition	range of variation
dimensionless salinity	x_1	([NaCl] − 3)/2	(−1, 1)
dimensionless pH	x_2	([pH] − 5.5)/2.5	(−1, 1)
dimensionless temperature	x_3	([T] − 30)/26	(−1, 1)

Table 2. Dependent Variables Used in This Study

dependent variables		
variable	nomenclature	units
surface tension (ST)	y_1	mN/m
emulsion volume (EV) gasoline/water	y_2	%
emulsion stability (ES) gasoline/water	y_3	%

The experimental data were analyzed by the Response Surface method with Statistica 7.0 software.

Study of the Synergistic Effects of Salinity, pH, and Temperature on the Surface-Active Properties of the Biosurfactant Produced by *L. pentosus*. Batch experiments were carried out in glass tubes containing 5 mL of biosurfactant and adjusted to the selected values of salinity, pH, and temperature, following the incomplete factorial design described above (see Table 1) for 2 h.

The dependent variables studied were the surface tension of the solutions under the established independent variables (y_1) as well as the emulsifying volume (EV) of the biosurfactant to stabilize gasoline/water emulsions (denominated y_2), and the gasoline/water emulsion stability (ES) (denominated y_3) was tested with gasoline.

Surface Activity Determination. The surface activity of biosurfactants produced by the bacterial strains was determined by measuring the surface tension of the samples with the ring method. The surface tension of PBS extract containing the biosurfactants produced by *L. pentosus* was measured using a KRUSS K6 Tensiometer equipped with a 1.9 cm Du Noüy platinum ring. Measurements were made on triplicate samples to increase the accuracy. The range of salinity was selected on the basis of the salinity of seawater. On average, seawater in the world's oceans has a salinity of about 3.5%.

Evaluation of the Emulsion Volume and Stability of Gasoline/Water Emulsion. The emulsion-stabilizing capacity of the biosurfactant produced by *L. pentosus* to stabilize gasoline/water emulsions was established according to Willumsen and Karlson[21] as the ability of the bioemulsifier to maintain at least the 50% of the original emulsion volume 24 h after formation. The relative emulsion volume was calculated according to Portilla-Rivera et al.[10] by mixing an equal volume (2 mL) medium containing the biosurfactant and the hydrocarbon, in this case gasoline. The solution was then shaken vigorously for 1 min and allowed to stand for 48 h. The relative emulsion volume (EV, %) and stability (ES, %) were then calculated with eqs 5 and 6, respectively.[22]

$$\text{EV (\%)} = \frac{\text{emulsion height (mm)} \times \text{cross-section area (mm}^2\text{)}}{\text{total liquid volume (mm}^3\text{)}} \times 100 \quad (5)$$

$$\text{ES (\%)} = \frac{\text{EV}_t}{\text{EV}_0} \times 100 \quad (6)$$

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where EV_t is the emulsion volume after 48 h and EV_0 is the emulsion volume at zero time.

RESULTS AND DISCUSSION

The experimental test conditions (expressed as coded variables) and the experimental data obtained for the dependent variables y_1−y_3 are shown in Table 3. The relationship between coded and uncoded variables was established by linear equations deduced from their respective variation limits, according to eq 7:[18]

$$x_i = \left(\frac{z_i - z_i^0}{\Delta z_i}\right)\beta_d \tag{7}$$

where Δz_i is the distance between the real value in the central point and the real value in the superior or inferior level of a variable, β_d is the major coded limit value in the matrix for each variable, and z^0 is the real value in the central point. Coded variables were then assigned values of −1, 0, and +1, corresponding to the lowest, central, and maximum limits of variation for each variable. The response surface obtained from the coded variables is therefore not influenced by the magnitude of each variable, which allows combination of the factors on a dimensionless scale.

Table 3. Operational Conditions Considered in This Study (Expressed in Terms of the Coded Independent Variables) and Experimental Results Achieved for the Dependent Variables y_1−y_3

	independent variables			dependent variables		
exp.	x_1	x_2	x_3	y_1	y_2	y_3
1	0	−1	−1	67.8	0	0
2	0	1	−1	55.2	45.93	100
3	0	−1	1	68.7	0	0
4	0	1	1	53.8	40.43	74.85
5	−1	−1	0	58.8	0	0
6	−1	1	0	55.1	45.25	76.93
7	1	−1	0	69.3	0	0
8	1	1	0	56.2	45.73	71.85
9	−1	0	−1	55.5	45.90	69.61
10	−1	0	1	60.2	21.53	62.94
11	1	0	−1	59.1	21.16	29.54
12	1	0	1	57.0	30.12	74.95
13	0	0	0	58.0	46.23	91.17
14	0	0	0	58.0	47.48	93.31
15	0	0	0	59.0	46.44	93.27

Synergistic Effects of Salinity, pH, and Temperature on the Surface-Active Properties of the Biosurfactant Produced by *L. pentosus*. At high pH (8), the salinity, within the range tested, almost did not affect the surface tension of the biosurfactant produced by *L. pentosus*, whereas at pH 3 and 3% salinity, the surface tension of the biosurfactant increased by 14 units and the biosurfactant almost lost its surface properties. However, at pH 5.5 and 5% salinity, the surface tension of the biosurfactant only increased by 3 units, showing that at intermediate pH, a salinity of around 5% has negligible effects on the surface properties of the biosurfactant (Table 3). Low pH (3) also had a strong negative effect on the emulsifying capacity of the biosurfactant, as the biosurfactant completely lost its emulsifying capacity (Table 3).

The coefficients and the significance of each coefficient (p values) for the variables y_1−y_3, corresponding to the dependent variables tested are shown in Table 4. Using these coefficients, equations can be created to determine the values of the dependent variables studied, within the ranges tested.

Equations 8−10 may serve, respectively, as proxies for calculating the surface tension (y_1) and emulsifying capacity (y_2) of the biosurfactant and the emulsion stability (y_3) of the emulsions stabilized by the biosurfactant. The equations for all of the dependent variables studied are shown below. Coefficients with p values >0.05 were ignored in the equations because they are not statistically significant.

$$y_1 = 58.33 + 1.5[\mathrm{NaCl}] - 5.54[\mathrm{pH}] - 2.35[\mathrm{NaCl}][\mathrm{pH}] - 1.7[\mathrm{NaCl}][\mathrm{T}] + 2.47[\mathrm{pH}]^2 \tag{8}$$

$$y_2 = 46.72 - 1.96[\mathrm{NaCl}] + 22.17[\mathrm{pH}] - 2.61[\mathrm{T}] + 8.33[\mathrm{NaCl}][\mathrm{T}] - 7.94[\mathrm{NaCl}]^2 - 16.02[\mathrm{pH}]^2 - 9.10[\mathrm{T}]^2 \tag{9}$$

$$y_3 = 92.58 - 4.14[\mathrm{NaCl}] + 40.45[\mathrm{pH}] + 13.02[\mathrm{NaCl}][\mathrm{T}] - 6.29[\mathrm{pH}][\mathrm{T}] - 19.92[\mathrm{NaCl}]^2 - 35.47[\mathrm{pH}]^2 - 13.40[\mathrm{T}]^2 \tag{10}$$

For variables y_1 and y_3, the most important independent variable was pH (x_2), followed by salinity (x_1), whereas temperature had a negligible effect on the surface-active properties. For this dependent variable, temperature had a greater effect than salinity on the emulsifying capacity of the biosurfactant produced by *L. pentosus*. These results are consistent with those reported by Abouseoud et al.[15] These authors studied the effect

Table 4. Regression Coefficients and Their Statistical Significance for Variables y_1−y_{14}

	y_1	p_{y1}	y_2	p_{y2}	y_3	p_{y3}
b_0	58.33	0.000033[a]	46.72	0.000068[a]	92.58	0.000058[a]
b_1	1.5	0.018019[a]	−1.96	0.014285[a]	−4.14	0.01074[a]
b_{11}	−0.95	0.086489	−7.94	0.001918[a]	−19.92	0.001021[a]
b_2	−5.54	0.001356[a]	22.17	0.000114[a]	40.45	0.000114[a]
b_{22}	2.47	0.014467[a]	−16.02	0.000472[a]	−35.47	0.000322[a]
b_3	0.26	0.327232	−2.61	0.008098[a]	1.70	0.059205
b_{33}	0.57	0.197842	−9.10	0.001463[a]	−13.40	0.002252[a]
b_{12}	−2.35	0.014757[a]	0.12	0.754239	−1.27	0.17368
b_{13}	−1.7	0.027645[a]	8.33	0.001609[a]	13.02	0.002203[a]
b_{23}	−0.57	0.184616	−1.37	0.05445	−6.29	0.009344[a]

[a]Significant coefficients ($p < 0.05$).

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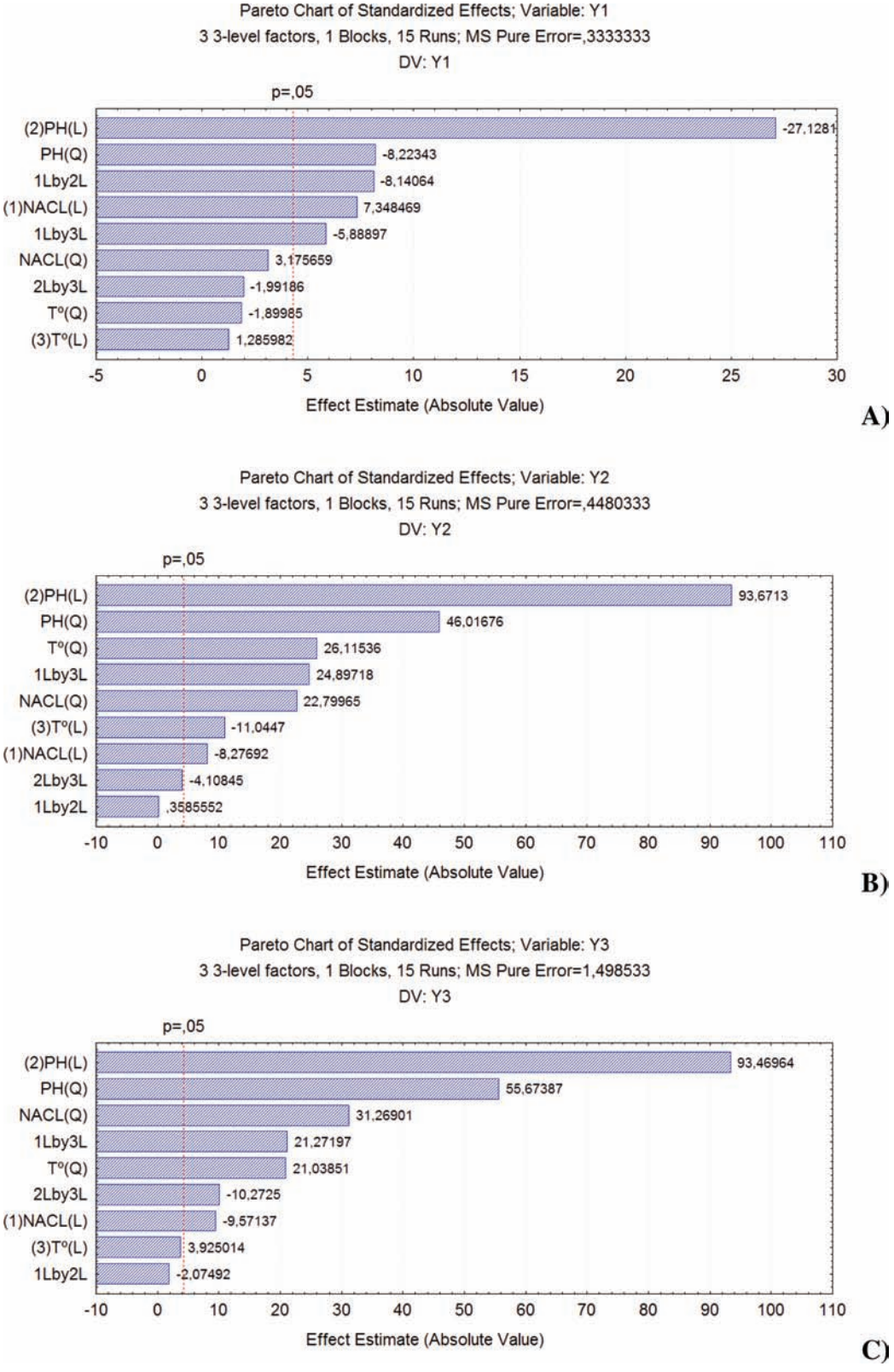

Figure 1. Pareto chart of standardized effects of variables y_1 (a), y_2 (b), and y_3 (c).

of pH and salinity on the emulsifying capacity of a biosurfactant produced by *P. fluorescens* and on solubilization of naphthalene by the biosurfactant and found that increases in pH had a positive effect on surface tension and emulsion stability, whereas salinity had a weak effect on surface tension and the emulsification index of the biosurfactant in the range tested (0−20%).

The Pareto chart of standardized effects of variable y_1 is shown in Figure 1a, whereas the Pareto charts of standardized effects of variables y_2 and y_3 after statistical treatment of the data are shown in Figure 1b,c, respectively; for all of the dependent variables studied, the most significant independent variable was pH, within the range tested.

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Figure 2 also shows the variation in y_1 with the most influential independent variables x_1 and x_2 (i.e., pH and salinity),

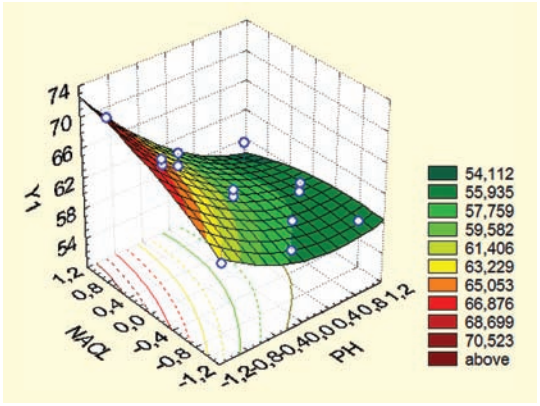

Figure 2. Variation in y_1 with pH and salinity at a fixed temperature of 30 °C.

within the ranges tested. Temperature, the least influential variable, was fixed at an intermediate value (x_3 = 30 °C). Salinities close to 1% and pH close to 5.5 resulted in the lowest surface tension values. However, as the salinity increased, the pH had to be increased to achieve the lowest surface tension, as low salinity and pH values exerted a synergistic effect on the surface tension of the biosurfactant. Moreover, Figure 3a shows the variation in surface tension values with temperature and salinity, with pH fixed at an intermediate value (x_2 = 5.5). In this case, the smallest reduction in surface tension occurred at values close to the lowest temperature and the lowest salinity evaluated. The same behavior was observed when the pH was fixed at the lowest values tested (x_2 = 3) (Figure 3b). However, when the pH was fixed at around 8, the lowest surface tension occurred at high temperature and salinity within the ranges tested (see Figure 3c). Consequently, at pH lower than 5.5, the salinity should not be higher than 1%; otherwise, the biosurfactant would lose it surface- active capacity.

Figure 4a shows the variation in y_2 with pH and salinity. The biosurfactant only exhibited high emulsifying activity at pH > 5.5. In this case, salinity, within the range tested, almost did not produce any change in the emulsify capacity of the biosurfactant. However, when the pH was fixed at 5.5 or 8, the maximum emulsify capacity of the biosurfactant can be achieved at salinities lower than 3% and temperatures below 30 °C. Figure 4b shows the variation in y_2 with temperature and salinity, with pH fixed at 5.5, and Figure 4c shows the variation in y_2 with temperature and salinity, with pH fixed at 8; similar behavior was observed in both cases, that is, a synergistic effect between low temperature and low salinity.

With regard to the emulsion stability (y_3), Figure 5a shows the variation in y_3 with pH and salinity. The biosurfactant only displayed high emulsion stability at pH > 5.5. As in the case of the emulsify capacity (y_2), the salinity, within the range tested, almost did not produce any change in the emulsifying capacity of the biosurfactant. However, when the pH was fixed at 5.5 or 8, maximum stability of the emulsions was achieved at salinities below 3% and temperatures lower than 30 °C. Figure 5b shows the variation in y_3 with temperature and salinity, with the pH fixed at 5.5; Figure 5b shows the variation in y_3 with temperature and salt concentration, with pH fixed at 8; in both cases, the same behavior was observed as for y_2.

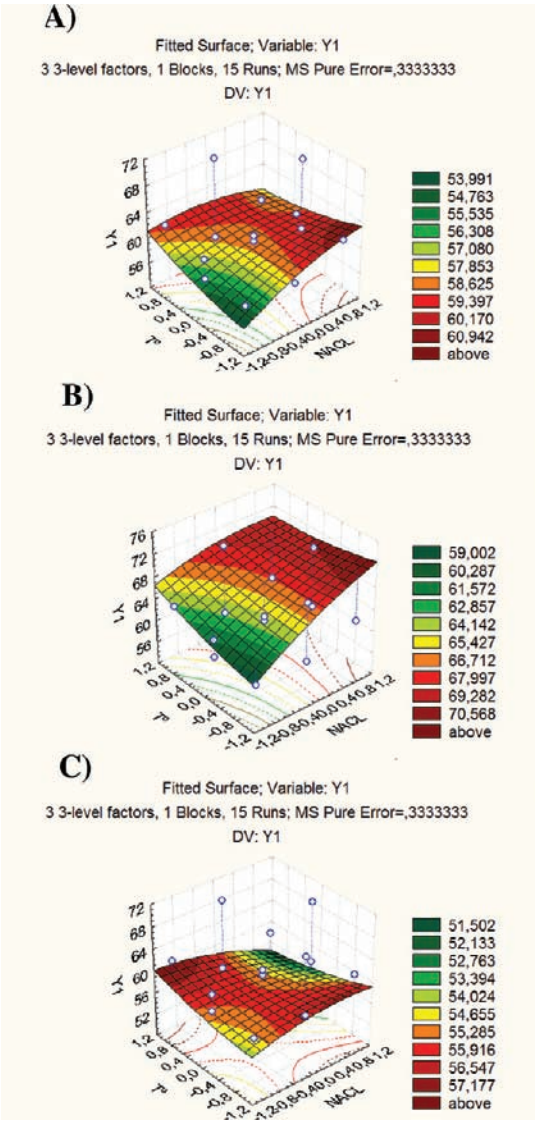

Figure 3. Variation in surface tension (y_1) values with temperature and salinity at pH (a) 5.5, (b) 3, and (c) 8.

Bharali et al.[12] studied the effect of pH, salinity, and temperature on the emulsifying activity of the cell-free supernatant and culture broth on *n*-hexadecane, using a biosurfactant produced by the thermophilic *Alcaligenes faecalis*. The effectiveness of the biosurfactant was found to be limited under both alkaline and neutral conditions. However, an increase in the emulsifying activity was observed at pH 8−12, and maximum emulsification occurred at pH 8−10. These authors also found that salinity of 4% and high temperature (121 °C) did not reduce the emulsifying activity of the biosurfactant produced by *A. faecalis*. It is important to note that these authors studied the effect of pH, temperature, and salinity separately, without taking any synergistic effects into account. In the case of the biosurfactant produced by *L. pentosus*, maximal reductions in surface tension and higher stability were achieved at pH around 8. However, these properties may change with salinity and temperature. For instance, at pH 8, salinity of approximately 1%, and temperature of approximately 4 °C, the model predicts surface tension values of 51.5 mN/m, whereas when the salinity is fixed at 5% and temperature above 30 °C, the model predicts surface tensions values higher than 57.2 mN/m. Moreover, for pH 8

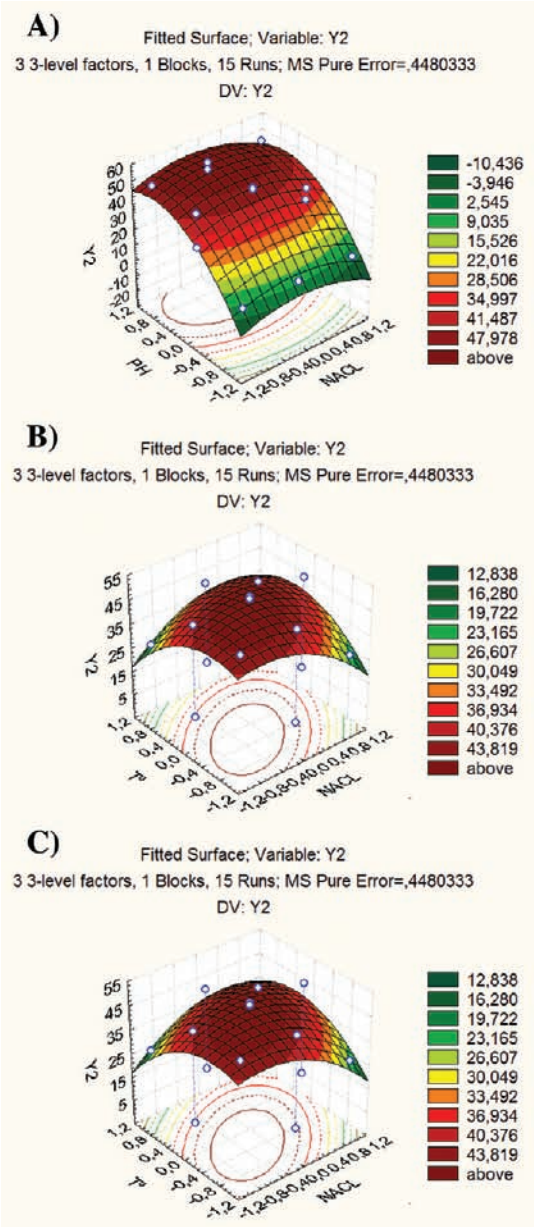

Figure 4. Variation in emulsifying capacity (y_2) of the biosurfactant produced by *L. pentosus* with pH and salinity at a fixed temperature of 30 °C (a). Variation in the emulsifying capacity (y_2) of the biosurfactant with the temperature and salinity at fixed pH values of (b) 5.5 and (c) 8.

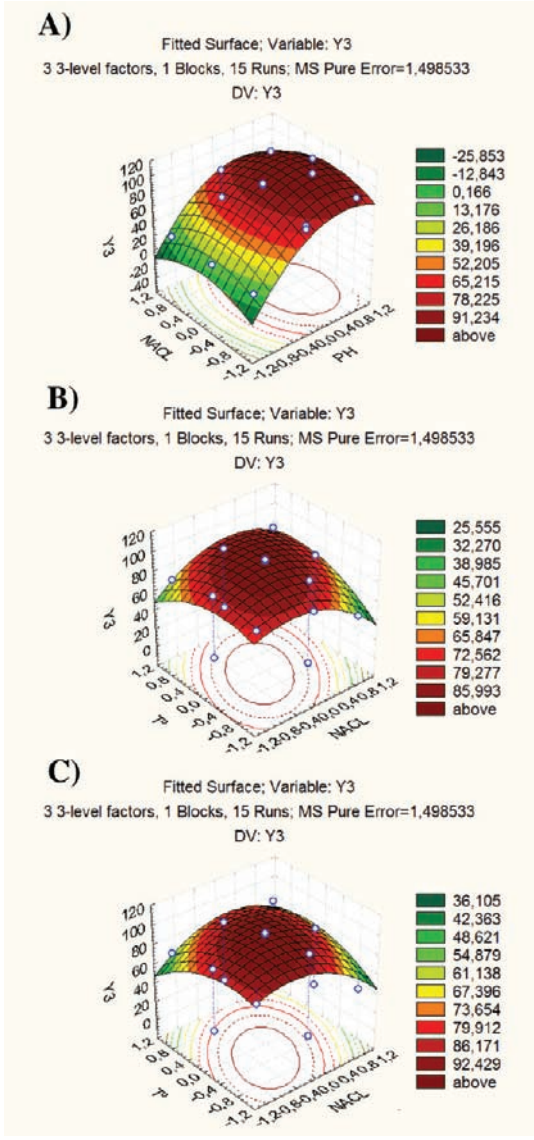

Figure 5. Variation in emulsifying stability (y_3) of the emulsion stabilized by the biosurfactant produced by *L. pentosus*, with pH and salinity, at a fixed temperature of 30 °C (a). Variation in emulsion stability (y_2) of the emulsion stabilized by the biosurfactant, with temperature and salinity, at fixed pH values of (b) 55 and (c) 8.

and salinity of approximately 3%, the model predicts surface tension values of around 55 mN/m, showing a negligible effect of temperature in the range tested (4−56 °C). On the other hand, we observed that the emulsifying capacity of the biosurfactant produced by *L. pentosus* and the emulsion stability are maximal in the range tested at temperatures below 30 °C and salinity below 3%, although it must be taken into account that salinity values higher than 3% reduce the emulsifying capacity and emulsion stability at below 30 °C. This behavior is maintained at pH between 5.5 and 8.

On the other hand, variables y_1, y_2, and y_3 yielded r^2 values of 0.91, 0.99, and 0.97 respectively. The coefficient of determination r^2 is used in the context of statistical models whose main purpose is the prediction of future outcomes on the basis of other related information. It is the proportion of variability in a data set that is accounted for by the statistical model and provides a measure of how well future outcomes are likely to be predicted by the model.[23] The statistical results obtained for the dependent variables (y_1−y_3) suggest that the model is appropriate for the data, as most of the factors and the interactions considered in the experimental design were significant at $p < 0.05$. Finally, Figure 6 shows the variation in observed vs predicted values for variables y_1−y_3; good agreement between experimental and theoretical data was observed.

In conclusion, the variable that most affected the surface-active properties of the biosurfactant produced by *L. pentosus* was pH, followed by salinity. Temperature had a negligible effect within the range tested (4−56 °C). Lower salinity allows use of the biosurfactant at lower pH, as a synergistic effect

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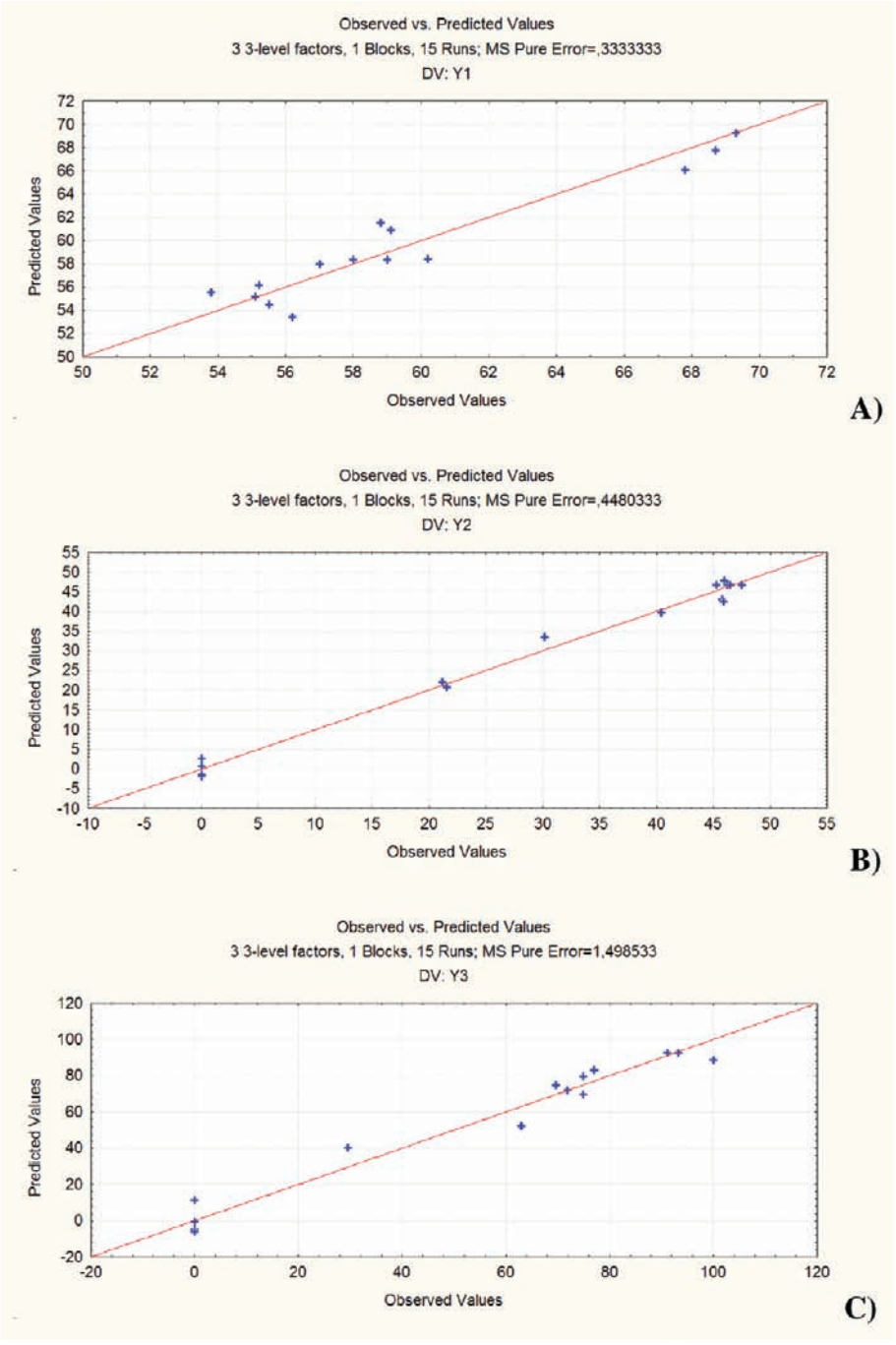

Figure 6. Variation in observed vs predicted values for variables (a) y_1, (b) y_2, and (c) y_3.

between low salinity and pH was observed. Moreover, the variable that had the greatest effects on the emulsifying capacity of the biosurfactant produced by *L. pentosus* was also pH, followed by temperature, although the emulsion stability was most affected by pH, followed by salinity. Important losses of emulsifying capacity were observed at pH below 5. The maximum emulsifying capacity of the biosurfactant and the stability of gasoline/water emulsions stabilized by the biosurfactant were achieved at temperatures below 30 °C, salinity below 3%, and pH higher than 5, and at low values, salinity and temperature had a synergistic effect on the emulsifying properties of the biosurfactant.

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Notes

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