

**OPTIMIZATION OF EXTRACTION CONDITIONS AND FATTY ACID  
CHARACTERIZATION OF *L. pentosus* CELL BOUND  
BIOSURFACTANT/BIOEMULSIFIER**

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## Abstract

**BACKGROUND:** There is currently much interest in the use of natural biosurfactants and bioemulsifiers, mainly in the cosmetic, pharmaceutical and food industries. However, there are no studies about the optimization of the extraction conditions of cell bound biosurfactants. In this work, a biosurfactant with emulsifier properties was extracted from *L. pentosus* cells, under different extraction conditions, and characterized.

**RESULTS:** During extraction, the most influential independent variables, on the emulsify capacity of biosurfactant, was the operation time, followed by temperature, and the salt concentration. Biosurfactant from *L. pentosus* was evaluated by fourier transform infrared spectroscopy and the composition in fatty acids were analyzed by gas chromatography-mass spectrometry. The hydrophobic chain of the biosurfactant from *L. pentosus* comprises 548 g kg<sup>-1</sup> linoelaidic acid (C18:2), 221 g kg<sup>-1</sup> oleic or elaidic acid (C18:1), 136 g kg<sup>-1</sup> palmitic acid (C16) and 95 g kg<sup>-1</sup> stearic acid (C18). In addition, emulsions of water and rosemary oil were stabilized with a biosurfactant produced by *L. pentosus* and compared with emulsions stabilized with polysorbate 20.

**CONCLUSIONS:** The optimum extraction condition of biosurfactant was achieved at 45 °C during 120 min and using 9 g kg<sup>-1</sup> of salt. In all the assays biosurfactant from *L. pentosus* yielded more stable emulsions and higher emulsion volumes than polysorbate 20.

**Keywords:** *Lactobacillus pentosus*, biosurfactant, fatty acids, rosemary emulsion.

## 1. INTRODUCTION

Lactic acid bacteria, which are generally recognized as safe (GRAS) by the U.S. Food and Drug Administration, are considered suitable for applications in the pharmaceutical, cosmetic

and food industries. These microorganisms have the ability to produce lactic acid and biosurfactants with emulsifying properties from agricultural residues.<sup>1,2</sup>

Surfactants have a wide range of applications e.g., chemically synthesized surfactants are used in the pharmaceutical, cosmetic, petroleum and food industries, because they help form emulsions by enhancing the solubility of hydrophobic compounds, such as essential oils, in water. However, the high concentrations of surfactants typically required to ensure complete microemulsification and formulation stability often result in unacceptably high residues and toxicity. Thus, many authors recommend the use of biosurfactants rather than chemically synthesized surfactants because the former are fully biodegradable and can be produced using renewable resources as substrates.<sup>3-7</sup>

Thus, biodegradable and non-toxic surfactants derived from starch were used by Rodríguez-Rojo et al.<sup>3</sup> to reduce the interfacial tension between water and rosemary oil. Moreover, in previous studies have proposed using grape marc or pruning wastes as carbon sources to produce biosurfactants from *L. pentosus*; however, to date, no chemical characterization of this biosurfactant has been done.<sup>1,2</sup>

Although, biosurfactants are significantly less toxic than synthetic petroleum based surfactants, studies on their use in cosmetic and pharmaceutical formulations are scarce because it would increase the cost of cosmetic and pharmaceutical products. Thus, most of the emulsified preparations such as creams and lotions are formulated using chemical surfactants such as sodium dodecyl sulfate (SDS) and polysorbate 20 (Tween 20).

On the other hand, an important step in the production of the biosurfactants is the extraction from the cells or from the fermentation media. Thus, some authors have proposed the purification of extracellular biosurfactants using various complex analytical technics;<sup>8</sup> whereas cell bound biosurfactant usually are extracted from microbial cells using buffer phosphate-NaCl solutions.

The aim of this study was to optimize the solid-liquid extraction conditions of the cell bound biosurfactant produced by *L. pentosus* and to investigate the fatty acid composition of the hydrophobic chain of this biosurfactant.

## 2. EXPERIMENTAL

### 2.1. Biosurfactant production

The biosurfactant used in the study was produced by *L. pentosus* using hemicellulosic sugars obtained from vineyard pruning wastes. These were first hydrolyzed to soluble sugars and then fermented by *L. pentosus* at 31 °C pH 5.85. The methodology has been described elsewhere.<sup>9-11</sup> Thus, the extraction of biosurfactant from cells was carried out at the beginning of the stationary phase, after 24 h of fermentation, once hemicellulosic sugars are depleted and maximum concentration of biomass can be obtained (9.1 g L<sup>-1</sup>).<sup>10</sup>

### 2.2. Extraction of cell bound biosurfactants from lactobacillus cells

Lactobacillus cells were recovered by centrifugation, washed twice in deionized water, and resuspended in 50 mL of phosphate-buffer saline (10 mmol L<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>/K<sub>2</sub>HPO<sub>4</sub>) using different salt concentrations (0 g kg<sup>-1</sup>, 9 g kg<sup>-1</sup> and 18 g kg<sup>-1</sup>), time of extraction (30 min, 120 min and 210 min) and temperature (25 °C, 45 °C and 65 °C). Thus, a Box-Behnken response surface methodology was applied for the extraction.<sup>12</sup> The range of independent and dependent variables studied are shown in **Table 1**. The standardized (coded) dimensionless independent variables used, with variation limits (-1, 1), were defined as  $x_1$  (0-18 g kg<sup>-1</sup>),  $x_2$  (30-210 min) and  $x_3$  (25-65 °C).

Thus, the quadratic function obtained for all three variables is described in **equation 1**.

$$y = \beta_0 + \beta_1x_1 + \beta_2x_2 + \beta_3x_3 + \beta_{12}x_1x_2 + \beta_{13}x_1x_3 + \beta_{23}x_2x_3 + \beta_{11}x_1^2 + \beta_{22}x_2^2 + \beta_{33}x_3^2 \quad \text{eq. (1)}$$

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

Maximum temperature during extraction was fixed at 65 °C because most of biosurfactants contain proteins and these are very unstable at high temperatures. Moreover, from an industrial point of view the utilization of higher temperatures would increase the production cost of biosurfactants reducing their commercial applications in comparison with chemical surfactants.

### *2.3. Determination of the biosurfactant concentration*

The biosurfactant concentrations ( $\text{mg L}^{-1}$ ) were then determined using a calibration curve. The calibration curve was calculated for a commercial biosurfactant produced by several *Bacilli* (surfactin) using different concentrations of biosurfactant solution, below the critical micelle concentration (CMC) with known surface tension. In this biosurfactant concentration range the decrease of surface tension is linear and it is possible to establish a relationship between the biosurfactant concentration and the surface tension.<sup>13</sup> To estimate biosurfactant concentration it was necessary to dilute the solution containing the biosurfactants to reach the critical micellar concentration.

### *2.4. Bioemulsifying study*

The emulsion stabilizing capacity of the surfactant (biosurfactant or polysorbate 20) to stabilize rosemary oil emulsions was established, in accordance with Willumsen and Karlson<sup>14</sup> as the ability of the emulsifier to maintain at least 50% of the original emulsion volume 24 h after formation. The relative emulsion volume (EV, %) and emulsion stability (ES, %) were calculated according to Portilla-Rivera et al.<sup>1</sup> and the solution (2 mL of solution containing the emulsifier with 2 mL of essential oil) was shaken vigorously on a vortex

shaker during 2 min. The mixture was then allowed to stand for 1 h, after which the EV and ES were measured at different time intervals (1.5 h, 3 h and 24 h). The EV and ES were then calculated using *equations 2 and 3* respectively.<sup>15</sup>

$$EV (\%) = \frac{\text{Emulsion height, mm} \times \text{Cross section area, mm}^2}{\text{Total liquid volume, mm}^3} \quad \text{eq. (2)}$$

$$ES (\%) = \frac{\% EV, \text{ at time } h}{\% EV, \text{ at } 0 \text{ h}} \times 100 \quad \text{eq. (3)}$$

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

To distinguish the continuous and the disperse phase of the emulsion, droplets of the emulsion were added to water or rosemary oil, and the behavior of the droplets was observed.

### 2.5. Morphology of oil/water emulsions

The emulsion droplets were observed under a Nikon Eclipse E800 optic microscope equipped with a Nikon camera. The emulsion was placed onto the microscope stage and observed through a 4X objective lens with ocular 10X where the total magnification was 40X. The droplets were measured (at  $20 \pm 2$  °C) and photographs taken using NIS Elements D2.30 SPI software (Nikon). This part of the study was carried out using emulsions that had been stored for 24 days.

### 2.6. Characterization of biosurfactant produced by *L. pentosus*

#### 2.6.1. Biochemical composition of biosurfactant

The carbohydrate content of the biosurfactant was determined by the phenol-sulfuric acid method, with D-glucose as a standard.<sup>16</sup> The protein content was determined by the method of Lowry et al.,<sup>17</sup> with bovine serum albumin as a standard. Lipid content was estimated by following the procedure of Folch et al.,<sup>18</sup> with cholesterol as a standard.

#### 2.6.2. Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy (FTIR) is very useful for identifying different types of chemical bonds (functional groups), and it can therefore be used to elucidate some components of an unknown mixture. The biosurfactant extract (1 mg) was ground with 10 mg of potassium bromide, and pressed (7,500 kg for 30 s) to produce translucent pellets. Infrared absorption spectra were recorded on a Thermo Scientific, Nicolet 6700 FTIR system with a spectral resolution of  $4\text{ cm}^{-1}$  and wave number accuracy between  $400$  and  $4000\text{ cm}^{-1}$ . All measurements consisted of 32 scans, and a potassium bromide pellet was used as background reference.

### 2.6.3. Fatty acid characterization of biosurfactant by GC-MS

The fatty acids of *L. pentosus* biosurfactant were converted into their methyl esters (FAMES), then analyzed by gas chromatography on a Model Trace GC Ultra (Thermo Scientific) equipped with a programmed split/splitless injector, coupled to a mass spectrometer, model Trace DSQ (Thermo Finnigan), controlled by XCalibur 1.4 software.

Prior to the analysis, the biosurfactant was submitted to rotary evaporation, to remove the aqueous phase, and it was then dissolved in dichloromethane. The sample was dissolved in  $500\text{ }\mu\text{L}$  of methyl tertiary-butyl ether and  $250\text{ }\mu\text{L}$  of trimethylsulfonium hydroxide. Nonadecanoic acid methyl ester (C19:0 ME) was added as internal standard. A sample volume of  $1\text{ }\mu\text{L}$  was injected using a split mode, with the split ratio of 1:50. The FAMES separation was performed on a ZB-WAX column ( $60\text{ m} \times 0.25\text{ mm i.d.} \times 0.25\text{ }\mu\text{m}$  film thickness) using an oven temperature gradient of  $60\text{ }^\circ\text{C}$  for 2 min, then  $60\text{-}200\text{ }^\circ\text{C}$  at  $10\text{ }^\circ\text{C min}^{-1}$ , held for 27 min, then to  $240\text{ }^\circ\text{C}$  at  $5\text{ }^\circ\text{C min}^{-1}$  and finally held for 20 min. Helium was used as carrier gas with a flow rate of  $1\text{ mL min}^{-1}$  and the temperature of both injector inlet and the transfer line of detector was set at  $250\text{ }^\circ\text{C}$ .

The mass spectras were obtained using a mass selective detector under electron impact ionization at a voltage of 70 eV and data acquisition was done at a scan rate of  $1.3459\text{ s}^{-1}$  over

an  $m/z$  range of 40-400. The software used to process peak areas was Xcalibur (version 1.4). FAMES were identified using a mass spectra library supplied with the GC-MS system and by comparison of retention times and mass spectra of a FAME standard mix (Supelco 37 Component FAME Mix: 10 mg mL<sup>-1</sup> of the FAME reference standard mix in methylene chloride, Sigma-Aldrich) injected in the same conditions.

### 2.7. Statistical analysis of the data

The experimental data were analysed by the response surface method, with Statistic 7.0 software (Statistic for Windows, Stat Soft, Inc, USA).

## 3. RESULTS AND DISCUSSION

### 3.1. Evaluation of the extraction conditions on the emulsifying properties of the biosurfactant produced by *L. pentosus*

Biosurfactants produced by microorganisms are emerging as an alternative source of stabilizers for the rapid synthesis of emulsions as well as nanoparticles.<sup>19</sup> Thus, the present study is focused on the ability of biosurfactants produced by *L. pentosus*, using vineyard pruning waste as carbon source, to stabilize rosemary oil-in-water emulsions.

An incomplete factorial design was used to study the effect of extraction conditions (salinity- $x_1$ , time- $x_2$  and temperature- $x_3$ ) on the emulsifying properties of the biosurfactant produced by *L. pentosus*. The dependent variables studied were the EV of rosemary oil-in-water emulsions at three different times (variables  $y_1$ ,  $y_2$ ,  $y_3$ ) and the ES of these emulsions at two different times (variables  $y_4$  and  $y_5$ ) (see **Table 1**).

The experimental data obtained for EV and ES (i.e. variables  $y_1$  to  $y_5$ ) of the 15 emulsions formulated with rosemary oil and water, and stabilized with the biosurfactant extracted from *L. pentosus*, are shown in **Table 2**. The concentration of biosurfactant in the emulsions was different depending on the extraction conditions.

The sequence of the experimental work was randomly established to limit the influence of systematic errors on the interpretation of results. Experiments 1-12 allowed calculation of the regression coefficients, whereas experiments 13-15 were replicates in the central point of the design and were used to estimate the influence of the experimental error.

The significance of each coefficient was determined by  $p$ -values for the variables  $y_1$  to  $y_3$ , corresponding to EV at 1.5 h; 3 h and 24 h respectively, and for variables  $y_4$  to  $y_5$  corresponding to the ES after 3 h and 24 h of emulsion formation (**Table 3**). Using these coefficients, equations can be created to determine the values of the dependent variables studied within the ranges tested. For each dependent variable, the coefficients included in **Table 3** can be replaced in *equation 1* and used to determine the values of each dependent variable under different extraction conditions (independent variables), always within the range tested. However, the most important application of the *equation 1* is the calculation of the optimal conditions for the extraction of biosurfactant based on the values of the dependent variables studied (EV and ES of the emulsion). Thus, extraction with 9 g kg<sup>-1</sup> of NaCl for during 3.5 h at 65 °C yielded maximum EV values, although good results were also obtained at 45 °C.

All the coefficients are statistically significant ( $p < 0.05$ ), indicating that all variables related to the extraction process of the biosurfactant affect the capability of the biosurfactant to act as an emulsifier (**Table 3**).

The most influential independent variables, in the range tested for the dependent variable studied, were time of extraction ( $x_2$ ), followed by temperature ( $x_3$ ), and the salt concentration ( $x_1$ ), as deduced from the absolute values of coefficients (**Table 3**).

**Figure 1** shows the variation in EV for variables  $y_1$  to  $y_3$  with the following revealed as the most significant variables: time of extraction ( $x_2$ ) and temperature ( $x_3$ ), with the percentage of salt (the least significant variable) fixed at intermediate values ( $x_1=9$  g kg<sup>-1</sup>). Intermediate

conditions of extraction time and salt concentration ( $x_1=9 \text{ g kg}^{-1}$ ;  $x_2=120 \text{ min}$ ) and high temperature of extraction ( $x_3=65 \text{ }^\circ\text{C}$ ) yielded EV values of approximately 75.5%, 71.1% and 68.2% after 1.5 h, 3 h and 24 h respectively, whereas when all the independent variables were fixed approximately at intermediate values ( $x_1=9 \text{ g kg}^{-1}$ ;  $x_2=120 \text{ min}$ ;  $x_3=45 \text{ }^\circ\text{C}$ ), in the surface response can be observed that the maximum EV values obtained would be 66.2%, 62.3% and 59.9%, corresponding to 1.5 h, 3 h and 24 h, respectively.

The effect of temperature was more significant when EV was measured after shorter time intervals (**Figure 1A**) because when EV was measured after 24 h, the temperature of extraction can be decreased slightly without affecting the emulsifying capacity of the biosurfactant.

Higher concentrations of salt (within the range tested) increased the EV values in fresh emulsion. However, when the EV was evaluated after 24 h, the highest value (68.2%) was obtained with the biosurfactant extracted with the intermediate concentration of NaCl ( $9 \text{ g kg}^{-1}$ ).

ES indicates the extent to which changes in the physico-chemical properties of the emulsion take place over time.<sup>20</sup> When rosemary oil/water emulsions were stabilized with the biosurfactant, the ES values were about 97.2% in the presence of biosurfactant extracted under intermediate operational conditions ( $45 \text{ }^\circ\text{C}$ , 120 min and  $9 \text{ g kg}^{-1}$  NaCl). **Figure 2** shows the variation in the ES with the most significant independent variables (time- $x_2$ -and temperature- $x_3$ ), when the salt concentration ( $x_1$ ) was fixed at an intermediate value ( $9 \text{ g kg}^{-1}$ ). As the optimal conditions are in the middle of the range tested, the operational conditions selected for the extraction process were  $9 \text{ g kg}^{-1}$  salt,  $45 \text{ }^\circ\text{C}$  and 120 min.

Furthermore, **Table 4** shows the comparison of experimental and theoretical EV and ES values of emulsions containing the *L. pentosus* biosurfactant, extracted under different extraction conditions, after 24 h of emulsion formation.

Reasonable agreement was observed between the experimental and theoretical data predicted by *equation 1* for all the dependent variables assayed, with  $r^2$  values of 0.81, 0.82, 0.80, 0.81 and 0.76 for  $y_1$  to  $y_5$  respectively.

### *3.2. Morphology of rosemary oil/water emulsions stabilized by biosurfactant produced by L. pentosus*

In order to define the emulsions as oil-in water (O/W) emulsions or water-in oil emulsion (W/O), droplets of emulsions stabilized by the biosurfactant or by polysorbate 20 were added to water or rosemary oil. The droplets of emulsion were very quickly solubilized in water, whereas when the emulsion droplets were added to rosemary oil they remained insolubilized. This fact demonstrates that the emulsions formulated with the biosurfactant or polysorbate 20 consisted of oil-in-water emulsion (O/W), in which the oil phase is dispersed as globules throughout an aqueous continuous phase. Thus, the emulsion can be referred to as an O/W emulsion. If the oil phase was the continuous phase, the emulsion would be referred to as a W/O emulsion, and in this case the droplets of emulsion would be soluble in rosemary oil. Thus, pharmaceutical products range from lotions of relatively low viscosity to semi-solid creams. Pharmaceutical W/O emulsions are typically used for external applications. In this case, the consistency of rosemary oil/emulsion stabilized with biosurfactant was similar to a cream that maintained its consistency over time, whereas the rosemary oil/emulsion stabilized with polysorbate 20 was less consistent and more unstable. Rosemary oil/water emulsions stabilized with polysorbate 20, at the critical micelle concentration at 21 °C (CMC=98.7 mg L<sup>-1</sup>), yielded EV values of 30.2-11% after 1.5-24 h of emulsion formation, whereas emulsions stabilized with the biosurfactant (CMC=2 mg L<sup>-1</sup> measured as surfactin equivalents following the protocol of Vecino et al.<sup>21</sup> at 25 °C) yielded EV values of 61-60% after 1.5-24 h of emulsion formation, whereas after 24 days, emulsions stabilized with the biosurfactant or polysorbate 20 yielded EV values of 55.5% and 5%, respectively.

Photographs of a rosemary oil/water emulsion stabilized with the biosurfactant or polysorbate 20, after storage for 24 days, and of a control emulsion formulated without biosurfactant are shown in **Figure 3**.

The size of the droplets has an important effect on the stability of the emulsion, its optical properties (e.g. lightness and color), rheology and also its sensory properties (e.g. creaminess).<sup>20</sup> The biosurfactant was able to stabilize the dispersed droplets of rosemary oil in water, whereas polysorbate 20 yielded very unstable emulsions (**Figure 3**). In fact, after 24 days it was very difficult to obtain picture microphotograph of the emulsion stabilized by polysorbate 20 because the droplets disappeared very quickly. Polysorbate 20 is a relatively stable non-toxic surfactant that is used as a detergent and emulsifier in a number of domestic, scientific, and pharmacological applications. However, in this case it was less effective than the biosurfactant in stabilizing rosemary oil/water emulsions.

The rosemary oil/water emulsion stabilized by the biosurfactant is a polydisperse emulsion composed by droplets with different particle size distribution, most of diameter < 100  $\mu\text{m}$  (**Figure 3**).

### 3.3. Characterization of the biosurfactant

The biosurfactant obtained under the optimal extraction conditions predicted in the previously described study (9 g kg<sup>-1</sup> of salt; 45 °C and 120 min) was chemically characterized. The chemical analysis revealed that the biosurfactant is a mixture of carbohydrate, protein and lipid in a combination of 1:3:6, respectively. FTIR spectroscopy was used to elucidate the molecular components of the biosurfactant extracted from *L. pentosus*. The FTIR spectrum of the biosurfactant produced by *L. pentosus* is shown in **Figure 4**.

The spectral analysis of the biosurfactant indicated the presence of peptide groups resulting from OH and NH stretching (wave numbers 3419 cm<sup>-1</sup> and 3290 cm<sup>-1</sup>, respectively).

Moreover, the FTIR spectrum of the biosurfactant indicated the presence of protein-related weak bands; C=O bond at  $1644\text{ cm}^{-1}$  (amide I bond) and N-H bonds ( $1544\text{ cm}^{-1}$ , amide II bond). The absorbance band between  $2961\text{ cm}^{-1}$  and  $2856\text{ cm}^{-1}$ , as well as bands at around  $1456$ ,  $1403$  and  $1385\text{ cm}^{-1}$  denote the presence of C-H stretching corresponding to  $\text{CH}_2$  and  $\text{CH}_3$  groups of aliphatic chains. The band at  $1089\text{ cm}^{-1}$  (C-O stretch vibration in sugars) also indicates the presence of polysaccharides. According to the FTIR spectrum obtained, the biosurfactant is composed of carbohydrates, lipids and proteins. These results are consistent with the analytical data showing that the biosurfactant is a glycolipopeptide. The hydrophobic chain of biosurfactant is composed by lipids, whereas the hydrophilic chain is mainly composed of proteins.

Glycolipopeptide biosurfactants are not commonly described in the literature. Most biosurfactants are composed of a unique hydrophilic and hydrophobic chain. Thavasi et al.<sup>22</sup> found that the biosurfactant produced by *Corynebacterium kutscheri* was also composed of a mixture of carbohydrate, lipid and protein in a combination of 40%, 27% and 29%, respectively. *Corynebacterium kutscheri* produced a biosurfactant with high amount of carbohydrates in comparison with the biosurfactant obtained from *L. pentosus*. FTIR spectra analysis also indicated the presence of carboxylic acids, N-H/C-H bonds of protein and  $\text{CH}_2$ -C-H asymmetric vibrations thus confirming the presence of alkanes in the biosurfactant produced by *Corynebacterium kutscheri*.

**Table 5** shows the fatty acids contained in the biosurfactant produced by *L. pentosus*, as well as the retention time of each fatty acid. Thus, the retention time for palmitic acid was 24.3 min, whereas for stearic, elaidic or oleic, and linoleaidic acid was 32.1, 33.2 and 36.0 min respectively. Chemical analysis revealed that the major fatty acid contained in the *L. pentosus* biosurfactant was linoelaidic acid followed by oleic or elaidic acids. It was observed that the fatty acid chain of the biosurfactant was similar to the hydrophobic fraction of the

rhamnolipid produced by *Pseudomonas putida*, which is mainly composed of palmitic, stearic, oleic and linoleic fatty acids with similar carbon chain length that those found in the *L. pentosus* biosurfactant.<sup>23</sup>

Various authors, Kanlayavattanakul and Lourith,<sup>24</sup> have pointed out that the use of lipopeptides is increasing as a result of the extraordinary surface properties and diverse biological activities of these compounds, thus simplifying an enormous number of applications in the pharmaceutical, cosmetic and food industries. Lipopeptides are used in various pharmaceutical products because of their beneficial surface properties.

On the other hand, in a previous work it was proved that *Lactobacillus acidophilus* cells does not show differences in viability before or after PBS extraction of biosurfactants achieving values of  $0.9 \times 10^9$  colony-forming units (CFU) mL<sup>-1</sup> before and after biosurfactant.<sup>25</sup> Thus it can be speculated that the PBS solution containing the biosurfactant from *L. pentosus* has no other substances as contaminants and the use of this biosurfactant may be interesting in the formulation of pharmaceutical and cosmetic products.

#### 4. CONCLUSIONS

Intermediate conditions of extraction time, salt concentration and temperature, in the range tested, gave a biosurfactant/bioemulsifier solution with optima properties to stabilize rosemary oil/water emulsions.

The chemical characterization revealed that the biosurfactant produced by *L. pentosus*, using as carbon source vineyard pruning waste, is a glycolipopeptide composed of C:18 and C:16 fatty acids, which are often used in the formulation of cosmetic products. This biosurfactant proved more success than polysorbate 20 in stabilizing rosemary oil/water emulsions. Therefore, the biosurfactant evaluated in this work and obtained by lactic acid fermentation of hemicellulosic sugars from vineyard pruning waste, may be of commercial interest for

various applications: in the cosmetic industry, for pharmaceutical formulations, and even for food production, as a biodegradable emulsifier.

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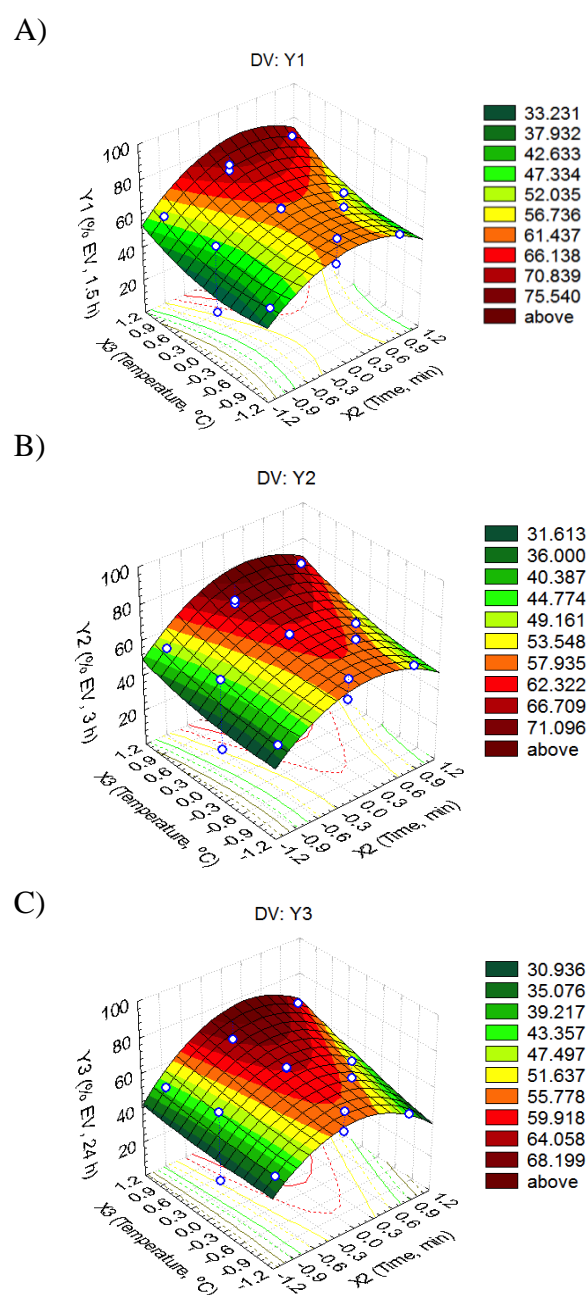
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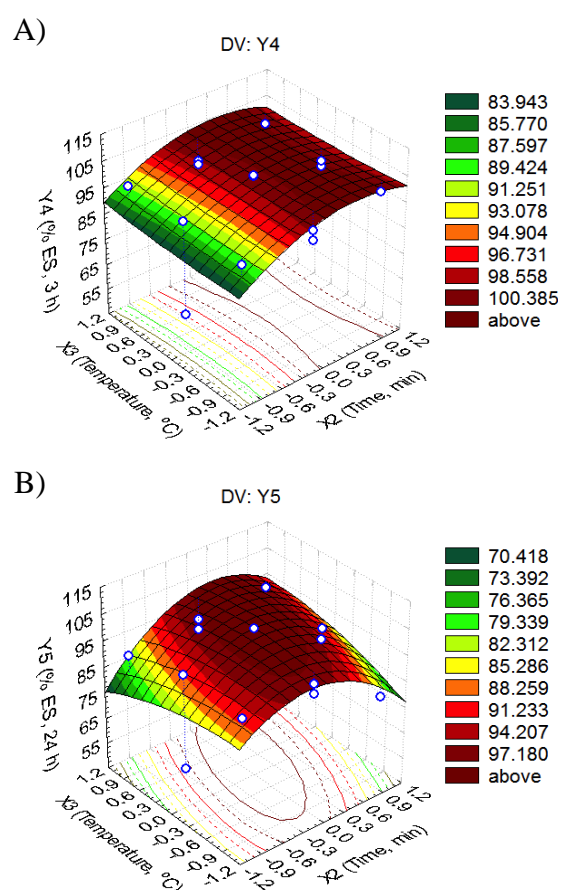
## FIGURE CAPTIONS

**Figure 1.** Dependence of a) EV after 1.5 h ( $y_1$ ); b) EV after 3 h ( $y_2$ ) and c) EV after 24 h ( $y_3$ ), with time and temperature, at intermediate concentration of salt.



**Figure 1**

**Figure 2.** Dependence of a) ES after 3 h ( $y_4$ ) and b) ES after 24 h ( $y_5$ ), with time and temperature, at intermediate concentration of salt.

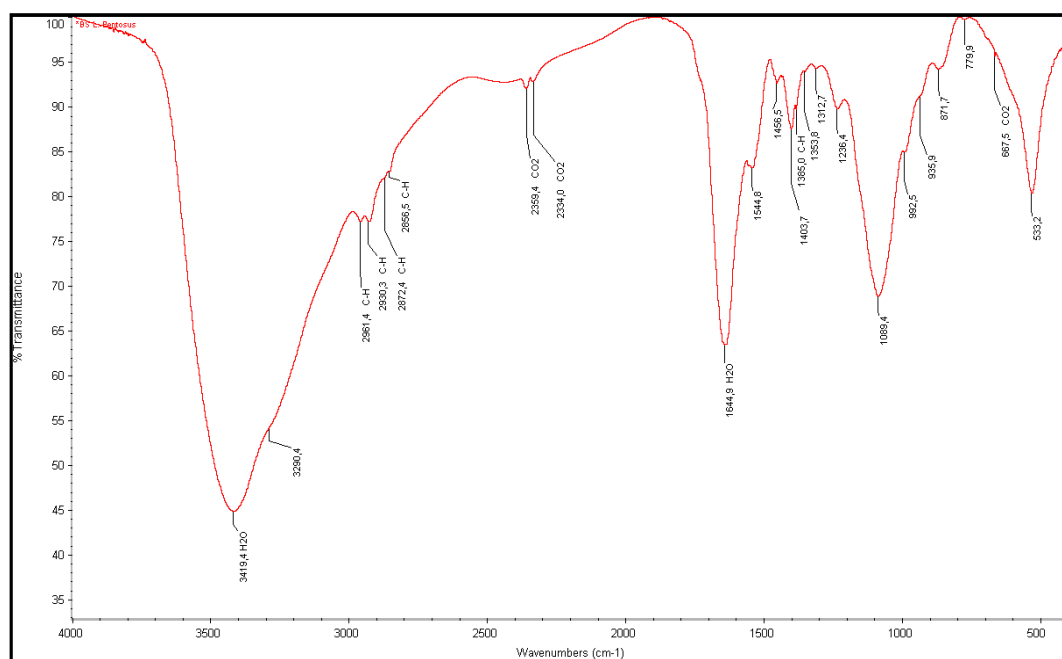


**Figure 2**



Figure 3

**Figure 4.** Fourier transform infrared spectrum of the biosurfactant produced by *L. pentosus*.



**Figure 4**

## LIST OF TABLES

**Table 1.** Independent and dependent variables used in the study.

**TABLE 1**

<i>Independent variables</i>			
<i>Variable</i>	<i>Nomenclature</i>	<i>Units</i>	<i>Range of variation</i>
Salinity	[NaCl]	g kg <sup>-1</sup>	0-18
Time	[t]	min	30-210
Temperature	[T]	°C	25-65
<i>Dimensionless, coded independent variables</i>			
<i>Variable</i>	<i>Nomenclature</i>	<i>Definition</i>	<i>Range of variation</i>
Dimensionless salinity	x <sub>1</sub>	$([\text{NaCl}] - 9)/9$	(-1,1)
Dimensionless time	x <sub>2</sub>	$([t] - 120)/90$	(-1,1)
Dimensionless temperature	x <sub>3</sub>	$([T]-45)/20$	(-1,1)
<i>Dependent variables</i>			
<i>Variable</i>	<i>Nomenclature</i>	<i>Units</i>	
EV <sup>1</sup> after 1.5 h	y <sub>1</sub>	%	
EV <sup>1</sup> after 3 h	y <sub>2</sub>	%	
EV <sup>1</sup> after 24 h	y <sub>3</sub>	%	
ES <sup>2</sup> after 3 h	y <sub>4</sub>	%	
ES <sup>2</sup> after 24 h	y <sub>5</sub>	%	

<sup>1</sup>Relative emulsion volume; <sup>2</sup>Emulsion stability

**Table 2.** Operational conditions used in this study (expressed as coded independent dimensionless variables: salinity  $x_1$ , time  $x_2$  and temperature  $x_3$ ) and results (in %) obtained for the dependent EV values after 1.5 h ( $y_1$ ), 3 h ( $y_2$ ) and 24 h ( $y_3$ ) and ES values after 3 h ( $y_4$ ) and 24 h ( $y_5$ ).

**TABLE 2**

Independent variable				Dependent variable				
Exp.	$x_1$	$x_2$	$x_3$	$y_1$	$y_2$	$y_3$	$y_4$	$y_5$
1	0	-1	-1	37.4	34.0	34.0	90.9	90.9
2	0	1	-1	46.5	44.3	33.2	95.3	71.5
3	0	-1	1	51.2	47.3	43.3	92.4	84.7
4	0	1	1	70.2	66.7	63.2	95.1	90.0
5	-1	-1	0	7.3	3.6	3.6	50.0	50.0
6	-1	1	0	41.5	37.7	33.9	90.8	81.7
7	1	-1	0	54.7	50.8	50.8	92.9	92.9
8	1	1	0	52.1	48.6	45.2	93.4	86.7
9	-1	0	-1	63.7	55.7	55.7	87.5	87.5
10	-1	0	1	64.3	58.3	56.8	90.6	88.3
11	1	0	-1	46.3	42.4	42.4	91.6	91.6

12	1	0	1	68.0	60.4	56.7	88.9	83.3
13	0	0	0	60.9	61.0	60.1	100.0	100.0
14	0	0	0	60.6	60.4	60.7	100.0	100.0
15	0	0	0	60.1	60.9	60.9	100.0	100.0

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**Table 3.** Regression coefficients and their statistical significance for variables  $y_1$  (EV after 1.5 h),  $y_2$  (EV after 3 h),  $y_3$  (EV after 24 h),  $y_4$  (ES after 3 h) and (ES after 24 h)  $y_5$ .

**TABLE 3**

	$y_1$	$p_{y1}$	$y_2$	$p_{y2}$	$y_3$	$p_{y3}$	$y_4$	$p_{y4}$	$y_5$	$p_{y5}$
$b_0$	60.5	0.00001	60.7	0.00000	60.5	0.00001	99.8	0.00000	100.0	0.00000
	3	5*	7	9*	7	6*	8	2*	8	1*
		0.00066		0.00037		0.00068		0.00016		0.00006
$b_1$	5.54	5*	5.86	6*	5.64	1*	5.98	4*	5.88	5*
$b_1$	-	0.00115	-	0.00029	-	0.00059	-	0.00010		0.00005
$b_1$	6.19	2*	9.73	5*	8.86	8*	10.9	6*	-9.44	5*
							4			
$b_2$	7.46	0.00036	7.70	0.00021	5.48	0.00072	6.05	0.00016	1.44	0.00109
		6*		8*		2*		0*		2*
$b_2$	-	0.00018	-	0.00011	-	0.00014	-	0.00024	-	0.00003
$b_2$	15.4	5*	15.8	1*	18.3	0*	7.17	6*	12.82	0*
	4		6		3					
$b_3$	7.48	0.00036	7.04	0.00026	6.84	0.00046	0.20	0.11588	0.61	0.00609
		5*		1*		3*		2		0*
$b_3$	6.23	0.00113	3.17	0.00277	1.19	0.03150	0.70	0.02459	-2.98	0.00055
$b_3$		7*		9*		4*		3*		1*
$b_1$	-	0.00048	-	0.00031	-	0.00053	-	0.00011		0.00005
$b_2$	9.20	2*	9.08	4*	8.98	8*	10.1	5*	-9.50	0*
							0			

b <sub>1</sub>	0.00146	0.00173	0.00395	-	0.00537	0.00087
5.28	3.85	3.30	-2.27			
3	4*	8*	6*	1.47	7*	7*
b <sub>2</sub>	0.00660	0.00495	0.00161	-	0.06230	0.00017
2.48	2.28	5.18	6.22			
3	0*	4*	4*	0.41	3	7*

\* Significant coefficients (p<0.05).

**Table 4.** Experimental and theoretical EV and ES values obtained at different extraction conditions after 24 h of emulsion formation.

Extraction conditions		Biosurfactant concentration		EV <sub>24h</sub> (%)		ES <sub>24h</sub> (%)	
Sal t (g kg <sup>-1</sup> )	Time (min)	Temperature (°C)	(mg L <sup>-1</sup> )	Experimenta l	Theoretica l	Experimenta l	Theoretica l
0	120	25	9.87	55.7	43.7	87.5	78.9
0	120	65	13.76	56.8	50.8	88.3	84.7
18	120	25	9.49	42.4	48.4	91.6	95.2
18	120	65	11.40	56.7	68.7	83.3	91.9
9	120	45	10.72	60.1	60.6	100.0	100.1

**Table 5.** Retention time and relative abundance of fatty acids in the biosurfactant produced by *L. pentosus*.

**TABLE 5**

<b>Retention Time (min)</b>	<b>Fatty acids</b>	<b>Formula</b>	<b>Relative Abundance (g kg<sup>-1</sup>)</b>
24.3	Palmitic acid	C <sub>16</sub> H <sub>32</sub> O <sub>2</sub> (C16)	136
32.1	Stearic acid	C <sub>18</sub> H <sub>36</sub> O <sub>2</sub> (C18)	95
33.2	Oleic or Elaidic acid	C <sub>18</sub> H <sub>34</sub> O <sub>2</sub> (C18:1)	221
36.0	Linoelaidic acid	C <sub>18</sub> H <sub>32</sub> O <sub>2</sub> (C18:2)	548