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# Production, characterization and kinetic model of biosurfactant produced by lactic acid bacteria

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ABSTRACT

*Background:* Biosurfactants are surface active molecules produced by microorganisms which have the ability to disrupt the plasma membrane. Biosurfactant properties are important in the food, pharmaceutical and oil industries. Lactic acid bacteria can produce cell-bound and excreted biosurfactants.
*Results:* The biosurfactant-producing ability of three Lactobacillus strains was analyzed, and the effects of carbon and nitrogen sources and aeration conditions were studied. The three species of lactobacillus evaluated were able to produce biosurfactants in anaerobic conditions, which was measured as the capacity of one extract to reduce the surface tension compared to a control. The decreasing order of biosurfactant production was *L. plantarum*>*Lactobacillus* sp.>*L. acidophilus*. Lactose was a better carbon source than glucose, achieving a 23.8% reduction in surface tension versus 12.9% for glucose. Two complex nitrogen sources are required for growth and biosurfactant production. The maximum production was reached at 48 h under stationary conditions. However, the highest level of production occurred in the exponential phase. Biosurfactant exhibits a critical micelle concentration of 0.359 ± 0.001 g/L and a low toxicity against *E. coli*. Fourier transform infrared spectroscopy indicated a glycoprotein structure. Additionally, the kinetics of fermentation were modeled using a logistic model for the biomass and the product, achieving a good fit ($R^2$ > 0.9).
*Conclusions: L. plantarum* derived biosurfactant production was enhanced using adequate carbon and nitrogen sources, the biosurfactant is complex in structure and because of its low toxicity could be applied to enhance cell permeability in *E. coli*.

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## 1. Introduction

Biosurfactants are a structurally diverse group of surface active molecules produced by microorganisms. They can accumulate on cellular surfaces or can be released into the extracellular medium. These amphiphilic molecules are preferred over their chemical homologues because of their biodegradability, low toxicity and efficiency in extreme temperature and pH conditions [1,2]. Biosurfactant properties such as emulsifying, antiadhesive and antimicrobial behavior are important in the food, pharmaceutical and oil industries where they are also used as hydrocarbon dissolution agents [3].

Lactic acid bacteria (LAB) include an extensive number of species involved in the fermentation of dairy products. Screening for biosurfactant production in diverse environments showed that around 30% of LAB strains are able to produce cell-bound and excreted biosurfactants. Medium development is an essential prerequisite to obtain higher productivity using any microbial strain. Therefore, it is important to know which nutrients and culture conditions are required to achieve higher productivity. There have been few studies on medium optimization for biosurfactant production. The aforementioned aspects should be investigated for each strain. Biosurfactants are able to form pores and disrupt the plasma membrane [4,5], and these characteristic have been helpful

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in facilitating bioremediation processes and increasing electricity production in microbial fuel cells [6,7].

However, it is important to consider the antimicrobial activity of biosurfactants to avoid inhibition of growth. The chemical structure of the biosurfactants produced by lactobacilli has been examined for different bacterial species. These biosurfactants can be made up of protein and polysaccharide fractions, glyco-lipids or xylopyranoside linked with octadecanoic acid [8]. Particular studies are needed to establish the chemical nature of the biosurfactants obtained [9]. On the other hand, the kinetic parameters of biosurfactants poduction are important to determine fermentation characteristics such as yields and growth rate. Few studies have been conducted on modelling the fermentation kinetics of biosurfactant production. *Lactobacillus* spp. [10] and *Bacillus licheniformis* STK were modeled with the use of logistic models [11,12], which could also be applied to other similar species.

The aim of the present study was to evaluate the potential of LAB to produce biosurfactants. The effect of the carbon and nitrogen source, as well as aeration, in biosurfactant production was studied and a characterization of the crude biosurfactant was performed. A kinetic study to stablish a model of growth, substrate consumption and biosurfactant production was also carried out.

## 2. Materials and methods

### 2.1. Screening for biosurfactant production

Three strains of LAB were evaluated in triplicate using the blood agar method at 30°C to determine their biosurfactant production potential: *Lactobacillus plantarum* ATCC 8014, *Lactobacillus acidophilus* NRRL B 4495, and *Lactobacillus* sp. (isolated from lactoserum). Hemolysis in blood agar was indicative of biosurfactant positive bacteria [13].

### 2.2. Selection of strain and culture conditions

A mix factorial design of experiments ($2^2 \times 3$ with three replicates) was performed to evaluate the effect of two carbon sources (glucose and lactose), two oxygenation conditions (aerobic and anaerobic) and the three LAB strains. In this way, the best conditions for growth and biosurfactant production could be determined. The culture medium employed with glucose was MRS broth (a medium for *lactobacilli* strains) [14], containing (per liter), 10 g peptone, 8 g beef extract, 4 g yeast extract, 20 g glucose, 2 g $K_2HPO_4$, 2 g ammonium citrate, 5 g sodium acetate, 0.2 g $MgSO_4$, 0.05 g $MnSO_4$, and 1 ml Tween 80. MRS that replaced glucose with lactose in the same concentration (MRS-Lac) was used to evaluate the effect of lactose. Aerobic experiments were carried out in 250 ml shake flasks containing 100 ml of medium, while anaerobic assays were done in 100 m shake flasks containing 100 ml of medium so that the head space was minimal. The culture conditions were 30°C and 120 rpm. Biomass, substrate and biosurfactant production were analyzed after 72 h of culture. ANOVA and LSD (Least Significant Difference) tests were performed to determinate the significance of factors and identify the best conditions of growth and biosurfactant production. Statgraphics Centurion XVIII software was used for analysis.

### 2.3. Effect of lactose concentration and nitrogen sources on biosurfactant production

The evaluation of how lactose concentration affected biomass and biosurfactant production was carried out using *L. plantarum*. The effect of lactose was evaluated using three concentrations (10, 20, 30 g/L). The influence of different nitrogen sources in the MRS-Lac broth (peptone, meat extract, and yeast extract) was evaluated by single factor design of experiments in triplicate (Table 1) as described by Gudiña et al. [15], maintaining the total amount of nitrogen equal to the basal MRS broth (2.9 g/L). After 72 h, surface activity, biomass production and substrate consumption were determined. Assays were done at 30°C and 120 rpm. The process variables for the best culture were monitored over time in order to identify its kinetic characteristics.

### 2.4. Biosurfactant recovery and characterization

To isolate and characterize the biosurfactant, the D culture in Table 1 was selected. 100 ml of culture medium was centrifuged for 10 min at 5000 rpm (3773 G-Centrifuge SIGMA 2-16PK). To determine the extracellular biosurfactant, surface tension was measured in the supernatant. Pellets obtained from centrifugation were washed twice in demineralized water, resuspended in 100 ml of phosphate-buffered saline solution (PBS: 10 mM $KH_2PO_4$/$K_2HPO_4$, 150 mM NaCl, pH adjusted to 7.0) and left at room temperature for 24 h with gentle stirring to release the biosurfactant. Subsequently, the bacterial debris were removed by centrifugation (5000 rpm, 10 min 3773 G-Centrifuge SIGMA 2-16PK) and the remaining supernatant was filtered through a 0.22 μm pore size filter. To determine the biosurfactant bounded to the cell, surface tension was measured in the supernatant. In order to concentrate the surfactant for characterization, the supernatant was ultra- filtrated (ultracel 10 kDa membrane, Millipore) and washed three times with milli Q water. Finally, the solution was freeze dried (0.12 mbar, −80°C) by lyophilization. Once dried, the crude powder biosurfactant was stored at −20°C until it was characterized.

### 2.5. Analytical methods

Bacterial growth was determined by measuring the optical density at 600 nm and sugar (glucose and lactose) concentration was measured using the reducing sugars method at 540 nm [16]. The surface tension of the culture broth and PBS extract samples was measured in accordance with the Ring method, using a *du Nouy* ring-type tensiometer (Cole Parmer) equipped with a 1.9 cm gold ring. The surface tension was measurement at room temperature after immersing the gold ring in the solution for a while to attain the equilibrium. The instrument was calibrated by measuring the surface tension of distilled water. All the measurements were taken in triplicate, and an average value was used to express the surface tension [17,18]. Sterile PBS with a surface tension of 70.6 ± 0.6 mN/m at 20°C was used as a control. The drop collapse [19] and oil dispersion [9] methods were used as qualitative evidence to support the presence of surface activity. Biosurfactant concentrations (g/L) were determined using a calibration curve (surface tension (mN/m) = −78,819 concentration (g/L) + 70,112, $r^2$ = 0.95). The calibration curve was calculated for the biosurfactant produced by *L. plantarum* using different concentrations below the CMC concentration. In this range of biosurfactant concentrations, the decrease in surface tension is linear and it is possible to establish a relationship between the concentration of biosurfactant and the surface tension [10].

#### 2.5.1. Critical micelle concentration (CMC)

CMC is a measure of the concentration in which an amphiphilic component initiates to form micelles. Above this concentration no further effect is expected in terms of surface activity [20]. To determine the CMC, surface tension as function of the logarithm of the concentration was plotted, CMC can be found as the point at which the baseline of minimal surface tension intersects the slope where surface tension shows a linear decline [21]. Solutions of crude biosurfactant at different concentrations (0–0,8 g $l^{-1}$) in PBS buffer

**Table 1**
Composition of the different culture media prepared by replacing the nitrogen sources of the MRS-Lac medium with ammonium. "D" corresponds to the standard MRS-Lac medium.

| (g/L) | A | B | C | D | E | F | G | H | I |
|---|---|---|---|---|---|---|---|---|---|
| peptone | 10.0 | 0 | 0 | 10 | 10 | 10 | 0 | 0 | 10 |
| Yeast extract | 0.0 | 4 | 0 | 4 | 4 | 0 | 4 | 0 | 4 |
| Meat extract | 0.0 | 0 | 8 | 8 | 0 | 8 | 8 | 0 | 8.1 |
| Ammonium citrate | 24.8 | 40.1 | 31.9 | 2.0 | 17.5 | 9.3 | 24.6 | 47.4 | 0.0 |
| Total nitrogen | 2.9 | | | | | | | | |

(pH 7.0) were prepared and the surface tension of each sample was determined in triplicate as described above.

*2.5.2. Antimicrobial assays*

The antimicrobial activity of the isolated crude biosurfactant against *E. coli DH5α* was determined using the microdilution method in 96- well plastic tissue culture plates [22]. Dimethyl sulfoxide (DMSO), a recognized sulfured antibacterial, was used as a positive activity control, and surfactin®, a commercial biosurfactant, was used to compare the antimicrobial activity with the biosurfactant produced.

*2.5.3. Fourier transform infrared spectroscopy*

The spectroscopy measurements of the samples were taken using the Attenuated Total Reflectance (ATR) technique within a wave range of 4000.0 to 400.0 $cm^{-1}$, using a FTIR spectrophotometer (Shimadzu IRTracer-100). A sufficient amount of freeze-dried biosurfactant (5 mg) was used to cover the circular detector (1 cm in diameter). Each sample was scanned 24 times at a 4.0 $cm^{-1}$ resolution and a mirror speed of 2.8 mm $s^{-1}$. Before analyzing the sample of interest, a polystyrene standard provided by the equipment manufacturer was checked to verify that the equipment was working well.

*2.6. Kinetic model*

The biosurfactant production kinetic was mathematically modeled in batch mode following the equations proposed by Mercier et al. [23] for the production of lactic acid by LAB and applied by other authors for the production of biosurfactants [10,11] and other bioproducts [24]. The cell growth model is expressed by a logistic equation, which represents cell growth from the beginning of the log phase to the stationary phase (including the deceleration phase). The biomass balance (X in g/L) in a batch process using the logistic equation is written as follows (**Equation 1**).

$$\frac{dX}{dt} = \mu_{max}\left(1 - \frac{X}{X_{max}}\right)X \tag{1}$$

where t is time (h), $\mu_{max}$ is the maximum specific growth rate ($h^{-1}$) and $X_{max}$ is the maximum biomass concentration (g/L). Through the integration of Equation 1 with appropriate initial conditions ($X = X_o$ at t = 0), Equation 2 is obtained.

$$X = \frac{X_o e^{\mu_{max} t}}{1 - \frac{X_o}{X_{max}}(1 - e^{\mu_{max} t})} \tag{2}$$

Biosurfactant production was mathematically modeled by analogy with the microbial growth model using **Equation 3**.

$$\frac{dP}{dt} = P_r\left(1 - \frac{P}{P_{max}}\right)P \tag{3}$$

where, $P$ is biosurfactant concentration (mg/L), $P_{max}$ is maximun concentration of biosurfactant (g/L), and $P_r$ is the ratio between the volumetric rate of product formation and the product concentration ($h^{-1}$). The equation can be directly solved to give **Equation 4**.

$$P = \frac{P_o P_{max} e^{P_r t}}{P_{max} - P_o + P_o e^{P_r t}} \tag{4}$$

The rate of substrate ($S$) utilization is the sum of two terms representing the biosynthesis of the product and the growth of microbial cells. The maintenance of cells is not included because it is almost negligible for *Lactobacillus* [23]. This relationship can be mathematically expressed by **Equation 5**:

where, $Y_{P/S}$ is the product yield on the substrate utilized (mg/g), and $Y_{X/S}$ is the biomass yield on the substrate utilized (g/g).

$$-\frac{dS}{dt} = \frac{1}{Y_{P/S}}\frac{dP}{dt} + \frac{1}{Y_{X/S}}\frac{dX}{dt} \tag{5}$$

By integrating ($S = S_o$ at t = 0), **Equation 6** is obtained.

$$S = S_o - \frac{1}{Y_{P/S}}(P - P_o) - \frac{1}{Y_{X/S}}(X - X_o) \tag{6}$$

The proposed model was fitted to experimental data using commercial software (Solver from Microsoft® Excel 2016) and parameters were identified using nonlinear regression with the least-squares method. The objective function to be minimized (**Equation 7**), was defined as the summation of the square difference between the experimental and predicted values ($y_i$) of biomass ($i$ = 1), lactose ($i$ = 2) and biosurfactant ($i$ = 3). The optimization problem to be solved during the parameter identification is also shown in Equation 7, where $x$ is the vector of parameters to be identified, lb (lower bounds) is the vector of minimum acceptable values of the parameters, ub (upper bounds) is the vector of maximum acceptable values for the parameters and $F_{obj}$ is the objective function to be minimized. Regression Analysis was used to calculate the regression coefficients $r^2$.

$$F_{obj} = \sum_{i=1}^{3}\left(y_{iexp} - y_{ipred}\right)^2$$

$$\min F_{obj} \mathbf{lb} < \mathbf{x} < \mathbf{ub} \tag{7}$$

## 3. Results and discussion

*3.1. Screening strains for biosurfactant production*

All of the three *Lactobacilli* strains tested showed zones of clearing in the blood agar under both aerobic and anaerobic conditions, which means they have the ability to cause complete hemolysis (β-hemolysis) in the blood agar culture media. These results are consistent with those of other authors who have tested LAB [10]. The blood agar lysis method was employed in this study as it has been widely used for screening biosurfactant production Nevertheless, the blood agar lysis has shown both false positives and negatives because there are several compounds (e.g. virulence factors) other than biosurfactants that may cause hemolysis. For this reason, the evaluation of hemolytic activity must be considered as a preliminary test. Combining screening methods and measuring surface tension are recommended [13,25].

### 3.2. Selection of strain and culture conditions

As mentioned previously, the factorial mix design included the following factors *Lactobacillus* strains, carbon source and oxygen supply. It was carried out in 100 ml and results are shown in Table 2. According to ANOVA, when using surface tension reduction as a response variable, the Pareto chart (Fig. 1) shows that the three factors evaluated (strain, carbon source and aeration condition) had a significant impact. There was only one interaction between the aeration condition and strain because no surface tension reduction of *L. acidophillus* under aerobic conditions was detected.

No significant reduction of surface tension was observed in the three cell-free supernatant culture broths. If some biosurfactant was released into the culture medium, it was not detected. However, a significant surface tension reduction ($p < 0.05$) was found in all PBS extracted samples (except for *L. acidophillus* under aerobic conditions), indicating that the surfactant produced is cell bounded.

The LSD test for the carbon source revealed statistically significant differences between glucose and lactose ($p < 0.05$), with a higher reduction in surface tension in MRS-lac than MRS broth for the three strains in anaerobic conditions. Variations in the uptake of different carbon sources via different pathways have previously led to varying amounts of by-products in LAB metabolism, e.g., flavors such as diacetyl and acetoin, bacteriocins and biosurfactants. The results in this work reflect that phenomenon. It has been shown that changing glucose for lactose increased the amount of biosurfactant produced by *L. lactis* [26]. According to experimental results, lactose is better than glucose for biosurfactant production, which may be related to surfactant requirements for lactose metabolism. In most types of LAB, lactose needs to be taken up from the culture media with the help of specific permeases, and the presence of surfactant could favor this transport [27]. The replacement of glucose with lactose as a carbon source has been reported for several types of LAB [2,15].

A greater reduction in surface tension was observed for anaerobic conditions (8.6 ± 0.6 mN/m) than aerobic conditions (4.0 ± 0.6 mN/m), according to the LSD test ($p < 0.05$). This could be related with other studies where some metabolite production is promoted by anaerobic conditions. For example, for the production of lactic acid by *L. plantarum*, anaerobic conditions were favored, whereas aerobic conditions were favored for the production of acetic acid [28]. There was no significant growth or significant reduction in surface tension for *L. acidophillus* under aerobic conditions, even though the blood agar test suggested that surfactant was produced in aerobic conditions. This behavior could be explained by agitation generating high levels of oxygen that could be not optimum for this microaerophilic strain [29]. For the other two LAB strains tested in the present work, respiration did not affect growth since there was no statistically significant difference in final biomass between the two conditions (final biomass 4.2 ± 0.2 g/L and 4.5 ± 0.1 g/L for aerobic and anaerobic conditions, respectively, $p < 0.05$). In LAB, the production of additional energy (ATP) during the aerobic/respiratory lifestyle (due to the conversion of pyruvate into acetate) promotes growth and survival in the stationary phase. However, the production of $H_2O_2$ and the oxygen accumulation, as well as the capability to synthesize antioxidant enzymes, might change the growth behavior of strains and may not result in an increase in biomass even if the extra ATP is produced [28].

**Table 2**
Biosurfactant production measured as surface tension generated on phosphate-buffered saline solution (PBS). Surface tension of PBS = 70.6 ± 0.6 (mN/m).

| | Surface tension of biosurfactants extracted with PBS (mN/m)* | | |
|---|---|---|---|
| Substrate (20 g/L) | Strain | Aerobic | Anaerobic |
| Glucose | *Lactobacillus* sp | 68.2 ± 2.7 | 64.0 ± 3.2 |
| | *L. plantarum* | 62.9 ± 1.6 | 61.5 ± 3.3 |
| | *L. acidophillus* | 70.6 ± 0.6 | 66.1 ± 0.6 |
| Lactose | *Lactobacillus* sp | 67.9 ± 2.9 | 62.0 ± 3.7 |
| | *L. plantarum* | 60.2 ± 1.8 | 53.8 ± 1.3 |
| | *L. acidophillus* | 70.6 ± 0.6 | 64.7 ± 5.7 |

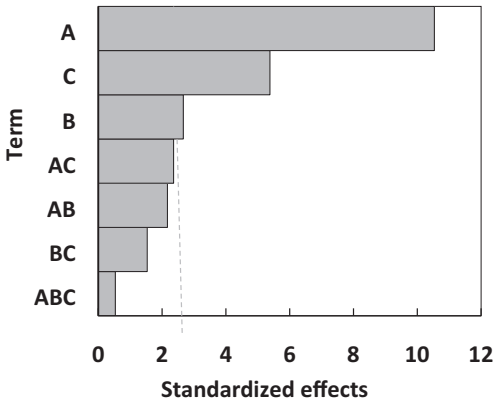


**Fig. 1.** Pareto Chart of the Standardized Effects (response is ST; α = 0.05). Response: Surface tension. Factors: Strain (A), carbon source (B), Aeration (C).

The LSD test for the microbial strain factor suggested that *L. plantarum* is the strain with the best biosurfactant production, with a surface tension reduction of 16.8 ± 1.6 mN/m (using lactose and anaerobic conditions). There are several reports describing the ability of different types of LAB, including *L. plantarum*, to produce biosurfactants. Madhu and Prapulla [18] also found that *L. plantarum* did not produce extracellular biosurfactant as indicated by a negligible difference in the surface tensions values of the cell free supernatant. Rodrigues et al. [20] found that *L plantarum* was a biosurfactant producer where the decrease in the surface tension exceeded 8 mN/m and Anukam and Reid [30] found that *L. plantarum* had the potential to inhibit the adhesion of uropathogens due to its ability to produce biosurfactants [18,30]. Often, LAB biosurfactant producers can act as probiotics. Biosurfactants play a crucial role in reducing the adherence capacity of several pathogens, which is a necessary step for biofilm proliferation and formation [31].

### 3.3. Effect of lactose concentration

The results of biomass production and surface tension reduction for *L. plantarum* for three levels of lactose are presented in Fig. 2. According to the LSD tests, for the two higher concentrations of lactose (20 and 30 g/L), final biomass and biosurfactant production were not statistically different ($p < 0.05$). Higher final biomass values of 38% and 37% were achieved with 30 g/L and 20 g/L of lactose, respectively compared to 10 g/L. Lower surfactant production and lower growth were observed for 10 g/L, indicating a possible limited carbon source at this concentration. This may be explained by the fact that although more substrate was available for 30 g/L, it was not consumed, as indicated by the fact that the biomass substrate yield for this concentration is 50% lower than for 10 g/L ($\boldsymbol{Y_{X/S}}$: 0.5,0.3 and 0.2 for 30 g/L , 20 g/L and 10 g/L of lactose respectively). These results are in accordance with other studies. For example, it was found that the concentration of lactose strongly affects cell growth and biosurfactant production. The highest biomass concentration (2.4 g/L) for the growth of *L. lactis* was achieved using MRS- Lac with 38.6 g/L lactose. Optimization of biomass concentration also positively affected biosurfactant production, and it

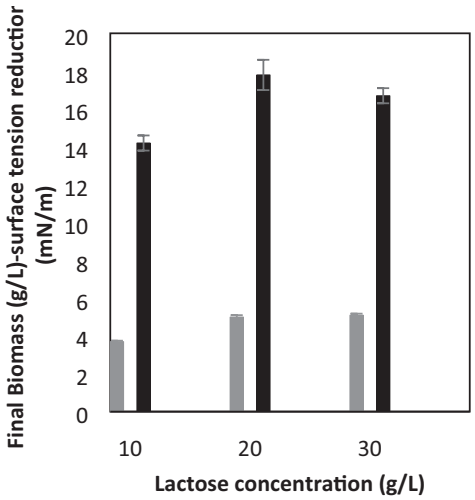


**Fig. 2.** Effects of lactose concentration on biomass (gray bars) and surface tension reduction (black bars) for surfactant produced from *L. plantarum* cultures compared to the PBS (72.2 mN/m). Results are expressed as mean ± standard deviations of values from triplicate experiments.

was found that biosurfactants are growth-associated [26]. For *L. paracasei,* 3.5 g/L of biomass and the highest surface tension reduction of 24.7 mN/m was achieved using 50 g/L of lactose [15].

### 3.4. Effect of nitrogen sources

Fig. 3 shows how changes in nitrogen sources affect biomass growth and biosurfactant production for *L. plantarum* using the same C/N ratio. When all the nitrogen present in the LAB medium was replaced by the equivalent amount of ammonium (medium H), no significant growth was observed (Fig. 3a). Low growth was observed in media with only one source of organic nitrogen (media A, B and C), while when using two (media E, F, G) or three organic nitrogen sources (media D and I) the highest biomass concentrations were achieved, without any statistically significant difference ($p < 0.05$).

Simple nitrogen sources like ammonium salts lack essential nutrients present in complex nitrogen sources. LAB require nucleotides, amino acids and vitamins because of the absence of various biosynthetic pathways of isoleucine, leucine and phenyl- alanine synthesis [32]. All three complex nitrogen sources are rich in amino acids. Meat extract and yeast extract have a higher diversity and number of free amino acids and vitamins than peptone because peptone is manufactured by acid or alkaline hydrolysis that may result in vitamin deterioration [33]. For this reason, *L. plantarum* may have preferred meat extract and yeast extract as individual nitrogen sources to obtain vitamins and free amino acids while benefiting from growth factors and purine and pyrimidine bases [34].

In terms of biosurfactant production, the G and I media yielded the highest values of surface tension reduction in the PBS extracts (18.5 mN/m on average), and there was no significant difference between them (Fig. 3b). These results are correlated with the highest biomass production in the G and I media.

### 3.5. Biosurfactant characterization

In the drop collapse assay, the collapsed droplets were observed using PBS extracts obtained from *L. plantarum*. The force or interfacial tension between the drop containing the surfactant and the parafilm surface was reduced and resulted in the spread of the drop, as reported for *L. acidophillus* [19] as an indicative of biosurfactant production. In the oil dispersion test, oil dispersion diameters of 3 ± 0.1 mm for water and 7 ± 0.5 mm for the extract were found. These two trials confirmed the production of biosurfactants in the *L. plantarum* cultures used in this work.

The results of the antimicrobial assay are presented in Fig. 4. DMSO, used as a positive control, presented complete inhibition of growth at a concentration of 12.5 g/L. When the culture was exposed at a concentration of 25 g/L of surfactant, cell growth of *E. coli* reached 64.5 ± 0.5% and 45.7 ± 2.7% using surfactin and biosurfactant produced from *L. plantarum* respectively, compared to the control without surfactant. In the rank of study, complete inhibition of growth was not observed using either commercial surfactin or biosurfactant from *L. plantarum.* There are few reports on the antimicrobial activity of biosurfactants isolated from *L. plantarum,* and those that do exist show low inhibition of *E. coli* ATCC 31075 [18]. Often the activity of biosurfactants against pathogens is related to a reduction in pathogen adhesion rather than direct antimicrobial activity or inhibition of cell growth [31].

The molecular composition of the purified and lyophilized biosurfactant used in this study was analyzed by Fourier transform infrared spectroscopy (Fig. 5). The most important bands were

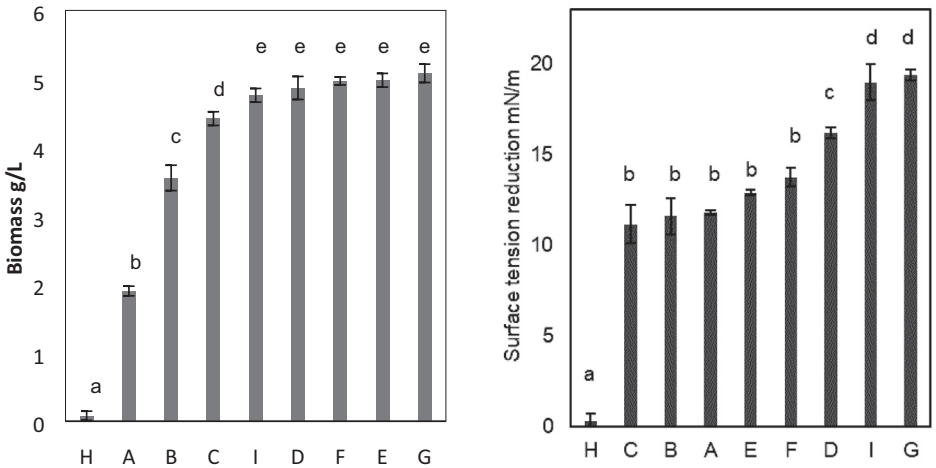


**Fig. 3.** Effects of nitrogen source on growth (a) and surface activity of the surfactant produced form *L. plantarum* compared against the PBS control (b), using 20 g/L lactose as a carbon source. Results are expressed as mean ± standard deviations of values from triplicate experiments.

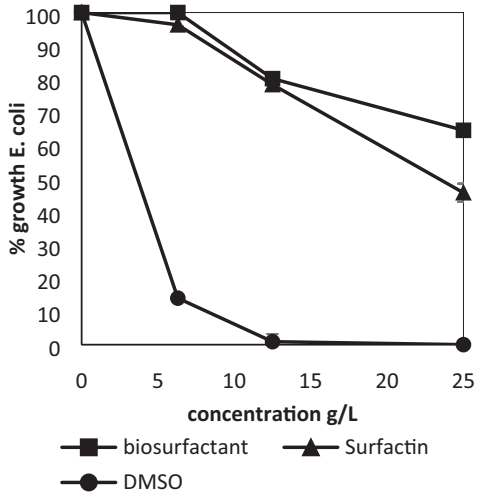


**Fig. 4.** Antimicrobial effect of crude biosurfactant isolated from *L. plantarum* cultures and commercial biosurfactin at different concentrations compared against *E. coli* growth.

located at 3280 $cm^{-1}$ (OH stretching), 2932 $cm^{-1}$ (CH band: CH2–CH3 stretching), 1639 $cm^{-1}$ (AmI band: CAO stretching in proteins), 1535 $cm^{-1}$ (N=O bending in proteins), indicating the presence of proteins in the sample analyzed, 1228 $cm^{-1}$ (PI band: phosphates), and 1060 $cm^{-1}$ (PII band: polysaccharides).

Analysis of the bands of the biosurfactant produced by *L. plantarum* in the present work demonstrated that the chemical composition is nonhomogeneous and is a glycopeptide with a phosphate fraction. Previously reported biosurfactant extracts from lactobacilli are composed of polysaccharides and lipid combinations (*L. hevelticus, L. pentosus*) [22,35], protein and polysaccharides (*L. plantarum, L. casei*) [8,18], or a combination of protein, polysaccharide and phosphate (*L. acidophilus*) [19,36]. The main physiological role of biosurfactants is to facilitate the uptake of water-immiscible substrates by lowering the surface tension at the phase boundary, emulsification, and enabling the microbial cells to adhere to the organic compounds [36]. Since biosurfactants are composed of hydrophilic and hydrophobic moieties, the organization of the functional groups of the biosurfactant produced in the present work could be phosphate and polysaccharide as the polar head, and a nonpolar structure of protein as the hydrophobic tail. However, further studies are needed to elucidate the biosurfactant structure [2].

Biosurfactant production, activity and composition of protein and polysaccharide fractions of glycoproteins are affected by environmental parameters such as composition of the medium, time, pH, temperature of incubation, inoculums volume, the growth phase of bacteria, aeration, and agitation speed [3,21,37]. Some of these factors were studied in the present work. The carbon source plays an important role in the growth and production of biosurfactants produced by microorganisms and varies from species to species [3]. The different carbon sources contribute to varying amounts of by-products. It can be hypothesized that the utilization of lactose as the main carbon sources induces the cells of *L. plantarum* to employ different metabolic pathways, and accordingly produces larger amounts of biosurfactant [25]. Nitrogen is the second most important supplement for the production of biosurfactants by microorganisms [3]. Complex nitrogen sources are essential for bacterial growth and lead to a higher generation of biosurfactants in some LAB species [15]. In terms of aeration, generally, cultures grown under respiratory conditions exhibited improved tolerance of some stresses (heat, oxidative stress, freezing) compared to those obtained in anaerobiosis. However, some mechanisms are not completely clear and the production of some metabolites, like biosurfactants, might be favored by a low concentration or absence of oxygen [28].

The results obtained for the biosurfactant produced by *L. plantarum* in MRS-lac and anaerobic conditions are presented in Fig. 6. They show a CMC of 0.359 ± 0.001 g/L and a surface tension of crude biosurfactant solution in PBS buffer of 50.0 mN/m. In order to use biosurfactants to facilitate cell transport by permeabilizing the cell membrane in processes such as bioremediation or in microbial fuel cells, several concentrations of biosurfactants under and above the CMC should be evaluated to elucidate the mechanism of interaction with cell membranes [7].

### 3.6. Kinetic model

Fig. 7 shows the experimental data as well as the predicted values calculated by Equation 2, Equation 4 and Equation 6 using the regression parameters listed in Table 3. All experiments show a kinetic pattern that is accurately described by the mathematical models, with $r^2$ values of 0.997, 0.995 and 0.988 (root-mean-

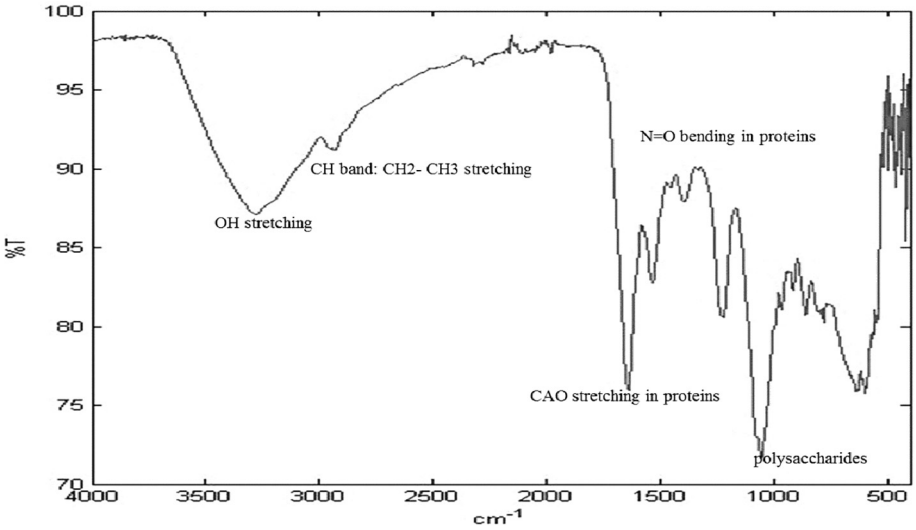


**Fig. 5.** The FTIR spectrum of the freeze-dried biosurfactant produced from *L. plantarum.*

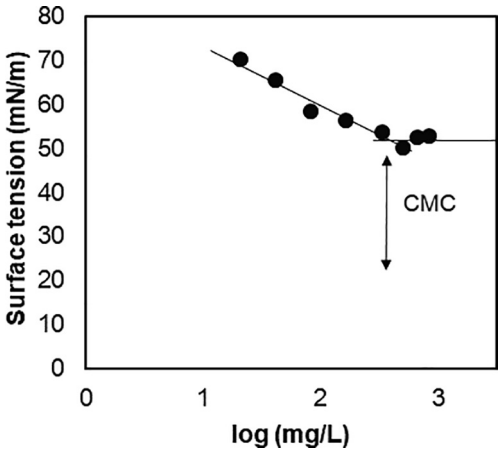


**Fig. 6.** Determination of Critical Mycelial Concentration (CMC) of the surfactant produced from *L. plantarum*.

square error -RMSE values of 0.134, 0.565 and 7.779) for lactose consumption, biomass and biosurfactant production, respectively. A time course kinetic study of *L. plantarum* on MRS- Lac showed a maximum biomass concentration of 4.5 g/L at 24 h. The production of biosurfactant was correlated with the exponential phase, which indicates that biosurfactants were produced as growth-associated metabolites. The use of a shorter incubation time (less than 72 h and reported extensively) is very important to increase productivity and reduce the production costs of biosurfactants [25]. The lactose degradation was 90% during the period of study.

Several mathematical models have been reported in the literature to express growth kinetics of LAB. However, few studies focus on the kinetics of biosurfactant fermentation in lactose. Although Monod based models are widely used to model bacterial growth, in some cases, especially with LAB, these models do not adjust well to the experimental values or they give unreal values of parameters. For this reason, several authors agree on the use of a logistic model to measure the growth kinetics of LAB, and they have found very good adjustment to experimental results. The Mercier Model is based on a generalized logistic equation for biomass and product kinetics. Although Mercier equations do not express an explicit relationship between biomass and product kinetics, equations (2) and (4) have the same mathematical structure association between growth and production [23].

Regarding the regression parameters listed in Table 3, the maximum specific growth rate ($\mu_{max}$) for *L. plantarum* is 0.47 $h^{-1}$ indicating that in the exponential phase the biomass could duplicate in 1.5 h. These values are in agreement with experimental results and with the range of specific growth rates for LAB. For example, $\mu_{max}$ was found to be 0.4 $h^{-1}$ for *L. pentosus* [10], and for *L. plantarum* growing in lactose and producing of lactic acid, $\mu_{max}$ was 0.38 $h^{-1}$at the optimum pH [38]. The maximum product ($P_{max}$: 199.9 mg/L) and biomass concentration ($X_{max}$: 4.15 g/L) values are comparable with the experimental values (209.6 mg/L of biosurfactant and 4.13 g/L of biomass), indicating good adjustment of the model. The maximum biomass is in the range of other LAB ($X_{max}$ 4.6–6.4 g/L) [10], but is very low compared with *Bacillus licheniformis* STK 01 which has a $X_{max}$ of 25.0 g/L [11]. Higher maximum product concentrations have been reported when compared with *L. plantarum* ($P_{max}$: 1.8 g/L for *L. pentosus* and $P_{max}$: 4.8 g/L for *B. licheniformis*). $P_r$ values reflect the activity of the microorganisms in terms of biosurfactant production. For *L. plantarum* $P_r$ was 0.15 $h^{-1}$, which is also low compared with other biosurfantact producers such as *L. pentosus* ($P_r$ = 0.5 $h^{-1}$) [10]. Values of substrate yields are important as indicatives of the distribution of substrate consumption for the growth and metabolite synthesis. The model value of $Y_{X/S}$ is in the range of other LAB (0.25–1.38 g/g) [10]. However, the $Y_{P/S}$ parameter fitted with the model (22.74 mg/g) is

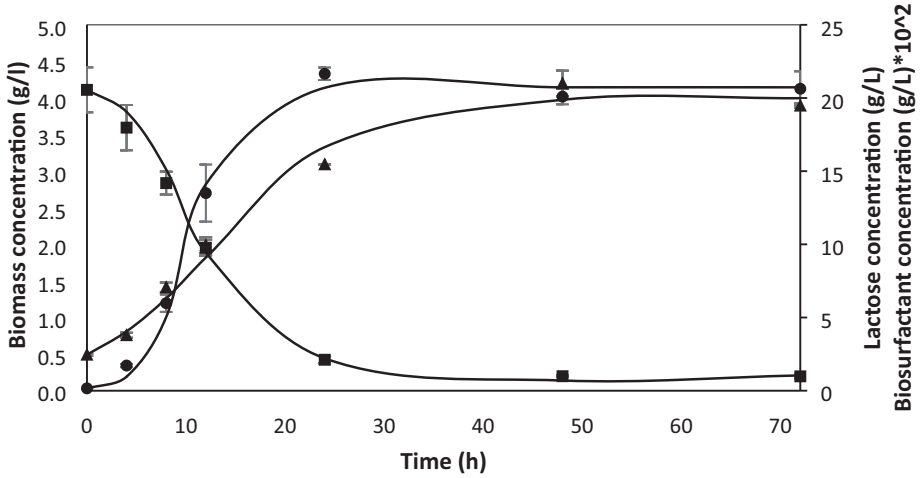


**Fig. 7.** Agreement of experimental and model-predicted values of the kinetic of *L. plantarum* during fermentations carried out with MRS-lac broth under anaerobic conditions (o) Biomass-g/L; (□) lactose-g/L; (△)biosurfactant-g/L*$10^2$.

**Table 3**
Estimated kinetic model parameters of biosurfactant production, biomass growth and lactose consumption for *L. plantarum*.

| Biomass growth | | | Biosurfactant production | | | | Lactose consumption | |
|---|---|---|---|---|---|---|---|---|
| $X_{max}$(g/L) | $\mu_{max}$ ($h^{-1}$) | $r^2$ | $P_{max}$(mg/L) | $P_r$ ($h^{-1}$) | $Y_{P/S}$(mg/g) | $r^2$ | $Y_{X/S}$(g/g) | $r^2$ |
| 4.15 | 0.47 | 0.995 | 199.9 | 0.15 | 22.74 | 0.988 | 0.34 | 0.997 |

lower than the yield for *L. pentosus* (90 mg/g) [10]. Operating conditions, bacterium species and strain are predominant factors in determining their specific biosurfactant production rates.

## 4. Conclusions

The results of this study have shown that the three strains of LAB: *Lactobacillus plantarum*, *Lactobacillus acidophillus* and *Lactobacillus* sp, produced cell bounded biosurfactant using MRS medium with glucose or lactose as a carbon source and under anaerobic conditions. Statistical analysis showed that *L. plantarum* was the best biosurfactant producer. The carbon source that best promoted biosurfactant production and growth was lactose. Anaerobic fermentation of *L. plantarum* for 48 h, less than the 72 h extensively reported, gave the minimum surface tension achieved in this study ($53.8 \pm 1.3\ Nm^{-1}$). Better growth and biosurfactant production were obtained when 20 g/L of lactose and two complex nitrogen sources, yeast extract (4 g/L) and malt extract (8 g/L) were used simultaneously. The antimicrobial test of the crude biosurfactant indicates low growth inhibition of *E. coli*, which was comparable with the commercial biosurfactant surfactin. FTIR analysis of the biosurfactant produced by *L. plantarum* demonstrates the presence of polysaccharide and protein fractions in its structure. The cell mass and biosurfactant concentrations could be accurately predicted using the modified logistic equation.

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## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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