Kinetic study of biosurfactant production by *Bacillus subtilis* LAMI005 grown in clarified cashew apple juice

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**Abstract**

In this work a low cost medium for the production of a biosurfactant by *Bacillus subtilis* LAMI005 and the kinetics of surfactin production considering the effect of initial substrate concentration were investigated. First, cashew apple juice supplementation for optimal production of biosurfactant by *B. subtilis* LAMI005 was studied. The medium formulated with clarified cashew apple juice and distilled water, supplemented with 1.0 g/L of (NH₄)₂SO₄, proved to be the best among the nutrients evaluated. The crude biosurfactant had the ability to decrease the surface tension of water to 30 dyne/cm, with a critical micelle concentration (CMC) of 63.0 mg/L. Emulsification experiments indicated that this biosurfactant effectively emulsified kerosene (IE₉₀ = 67%) and soybean oil (IE₉₀ = 64%). Furthermore, the emulsion stability was always very high. It was shown by biochemical analysis, IR spectra, that there is no qualitative differences in the composition of the crude biosurfactant from a standard sample of surfactin from *B. subtilis*.

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1. Introduction

Surfactants are surface active substances, consisting of a hydrophilic (polar) and a hydrophobic (nonpolar) part on its molecules [1,2]. They have the ability to reduce surface and interface tensions between liquids, solids, and gases, thus they allow them to mix or disperse readily as emulsions in water or other liquids [2]. Nowadays, the huge demand of surfactants is currently provided by chemical surfactants derived from petroleum, but these compounds have the problem of being toxic to the environment and non-biodegradable.

A biosurfactant is a surfactant produced extracellularly or as part of the cell membrane by bacteria, yeasts, and fungi from various substrates including sugars, oils, alkanes, among others [1]. They are amphiphilic compounds with considerable potential in commercial applications within various industries, such as health care and food processing industries, as well as enhancing oil recovery, crude oil drilling lubricants, bioremediation of water-insoluble pollutants [1,3,4]. Biosurfactant have advantages over its chemical counterparts in biodegradability and effectiveness at extreme conditions of temperature and pH. Furthermore, they have lower toxicity and can be produced from renewable and cheaper substrates [2,5–7]. However, the production of biosurfactant has some issues associated with the expensive costs of substrates and some inefficient product recovery methods [7]. Therefore, in order to enhance the industrial use of biosurfactant, the production cost should become competitive with the synthetic surfactants. A possible strategy to achieve this goal is to use alternative raw materials, such as agro-industrial residues such as cashew apple peduncle, as culture medium in submerge fermentation.

In the north coast of Brazil, the cashew agroindustry has an important role on local economy and the official estimate for the Brazilian cashew nut for 2011 was around 298 thousand of tons [8], which corresponds to more than 2.6 million tons of cashew apple. However, only 18% of the total peduncle is consumed in *natura* or processed industrially to produce different products from concentrated juice to desserts. It is estimated that about 15–20% of the pulp is exploited and 80% is wasted. Cashew apple is a pseudofruit rich in vitamin C, flavor and aroma, but the majority of cashew apples rot in the soil. Those facts, together with its rich composition (reducing sugar, vitamins and minerals salts), turns cashew apple juice (CAJ) into an interesting and inexpensive culture medium for biosurfactant production [9].

*Bacillus subtilis* is considered a suitable microorganism for biosurfactant production owing to the absence of pathogenicity, which permits the use of its products in food and pharmaceutical...
Table 1
Description of culture medium employed in this study using Bacillus subtilis LAM1005 in batch cultivation. TRS: total reducing sugars, mainly glucose and fructose, present in clarified cashew apple juice and TES: trace element solution, consisting in g/L by ZnSO4·7H2O: 10.95, FeSO4·7H2O: 5.0, MnSO4·H2O: 0.39, CuSO4·5H2O: 54, Co(NO3)2·6H2O: 0.25 and Na2B4O7·10H2O: 0.17.

<table>
<thead>
<tr>
<th>Culture medium</th>
<th>Nature of ingredients</th>
<th>C-source (g/L)</th>
<th>N-source (g/L)</th>
<th>TES (%)</th>
<th>Other (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>TRS (46.24)</td>
<td>(NH4)2SO4 (1.0)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>B</td>
<td>TRS (40.98)</td>
<td>(NH4)2SO4 (1.0)</td>
<td>0.1</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>C</td>
<td>TRS (45.65)</td>
<td>(NH4)2SO4 (1.0)</td>
<td>–</td>
<td>NaCl (2.7)</td>
<td>MgSO4·7H2O (0.6)</td>
</tr>
<tr>
<td>D</td>
<td>TRS (49.96)</td>
<td>(NH4)2SO4 (1.0)</td>
<td>–</td>
<td>Na2HP04·7H2O (7.2)</td>
<td>KH2PO4 (3.0)</td>
</tr>
<tr>
<td>E</td>
<td>TRS (43.79)</td>
<td>(NH4)2SO4 (1.0)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

industries [10]. Therefore, discovery of new strains and improvement in culturing methods of B. subtilis can provide a safe source of biosurfactant. Surfactin, one of the most effective cyclic lipopeptide biosurfactants produced by B. subtilis, can lower the surface tension of water from 72 to 27 dyne/cm and the interfacial tension of the water/n-hexadecane system from 43 dyne/cm to 1 dyne/cm [7].

Therefore, the aim of this work was to evaluate a low-cost culture medium, using cashew apple as carbon and energy source, to cultivate a new isolate of B. subtilis and to produce biosurfactant. The influence of juice concentration and its effect on biosurfactant concentration and surface activity were also investigated.

2. Materials and methods

2.1. Microorganism

B. subtilis LAM1005 used in this study was previously identified as a potential producer of biosurfactant [11] and it was isolated from the tank of chlorination at the Wastewater Treatment Plant on Campus do Pici (WWTP-PI) in the Federal University of Ceará, Brazil. It was identified by 16S rRNA gene sequence, which was deposited at Genbank with the accession number FJ413046. The strain was maintained on APGE medium (consisting of 15.0 g/L agar, 5.0 g/L peptone, 5.0 g/L glucose, and 2.5 g/L yeast extract) slants at 4 °C and transferred monthly as described in a previous work [11].

2.2. Clarified cashew apple juice preparation

Cashew apple juice (CAJ) was obtained by compressing the cashew apple (Anacardium occidentale L.). The juice was then centrifuged at 5000 x g for 20 min (BIO ENG, BE-6000), filtered using a 45 μm filter paper and clarified using gelatin (10–30%, w/v). The clarified cashew apple juice (CCAJ) was kept at –10 °C and it contains basically glucose, fructose, minerals and free amino acids as described before [9].

2.3. Culture medium

Clarified cashew apple juice (CCAJ) was diluted with water, supplemented with different nutrients (Table 1) and used in this study as culture medium for B. subtilis LAM1005 growth and biosurfactant production. The pH of the medium was adjusted to 7.0 with HCl (3 M) or NaOH (3 M) and it was sterilized at 110 °C for 10 min. When necessary, 0.1% (v/v) of a sterile trace element solution (consisting in g/L by ZnSO4·7H2O: 10.95, FeSO4·7H2O: 5.0, MnSO4·H2O: 0.39, CuSO4·5H2O: 54, Co(NO3)2·6H2O: 0.25 and Na2B4O7·10H2O: 0.17) was added after autoclaving.

2.4. Inoculum preparation

B. subtilis LAM1005 was inoculated (spread) onto APGE plates and incubated at 30 °C for 24 h. After this period, three loops full of the strain grown on APGE were transferred to 250-mL Erlenmeyer flask containing 50 mL of CCAJ, diluted with water up to 22.92 g/L of total reducing sugars (glucose and fructose), supplemented with 1.0 g/L of (NH4)2SO4 and 0.1% of trace element solution (consisting in g/L by ZnSO4·7H2O: 10.95, FeSO4·7H2O: 5.0, MnSO4·H2O: 0.39, CuSO4·5H2O: 54, Co(NO3)2·6H2O: 0.25 and Na2B4O7·10H2O: 0.17). The flasks were incubated in a rotary shaker (Tecnal-TE240, São Paulo, Brazil) at 180 rpm, 30 °C for 72 h. The OD of this culture was adjusted with sterile water to 0.1–0.2 at 600 nm, in order to assure that the same cell concentration was present at the seed culture.

2.5. Batch fermentation for biosurfactant production

Biosurfactant production was conducted in Erlenmeyer flask (250 mL) containing 50 mL aliquots of the culture medium. An aliquot of 5 mL (10%, v/v) of the seed culture was transferred to the Erlenmeyer flask and the experiments were carried out in a rotary shaker (Tecnal-TE240) at 180 rpm, 30 °C for 72 h. Samples were collected at defined intervals of time and submitted to analysis of biomass. Cells were removed by centrifugation at 10,000 × g for 20 min and the cell-free supernatant was assayed to determine its pH, carbohydrates, and surfactin concentrations. All assays were performed in duplicate and the results represent means ± standard deviations of the two independent experiments.

2.6. Analytical methods

2.6.1. Biomass content

Cell growth was determined by measuring the optical densities (OD) of samples, using a UV–vis spectrophotometer (20 Genesis, BR) at 600 nm. Biomass concentration, in g/L, was determined by calibration curve of dry weight (g/L) versus OD [11].

2.6.2. Carbohydrate concentration

Substrate concentration (glucose and fructose – Total Reducing Sugars, TRS) was measured by HPLC using a Waters high-performance-liquid chromatographer (Waters, Milford, MA) equipped with a refractive index detector (Model 2414, Waters), a Supelcogel C610H (30 cm × 7.8 mm) column and a Sigma–Aldrich pre-column (5 cm × 4.6 mm). Ultrapure water (Simplicity 185, Millipore, Billerica, MA) with 0.1% v/v of H3PO4 was used as solvent with a flow rate of 0.5 mL/min at room temperature (around 28 °C) and the sample size was 10 μL. The samples were identified by comparing the retention times with those of carbohydrates standards.

2.6.3. Emulsifying index

Emulsifying index was performed according to Cooper and Goldenberg [12], with slight modifications [13]: 2 mL of cell-free supernatant were added to 2 mL of kerosene, gasoline or soybean oil, and the mixture was vortexed for 2 min. After 24 h, the height of
emulsion layer was measured. The emulsification index (IE_{24}) was calculated using Eq. (1).

\[ \text{IE}_{24} (\%) = \frac{H_{\text{EL}}}{H_{\text{T}} \times 100} \]  

(1)

where \( H_{\text{EL}} \) is the height of the emulsion layer and \( H_{\text{T}} \) is the height of total solution. A control sample was prepared by using 2 mL of culture medium before inoculation instead of the cell-free supernatant.

2.6.4. Emulsification activity

Emulsification activity was determined according to Ciriglione and Carman [14], with slight modifications [11]. Samples of the supernatant free of cells (0.5 mL) were placed in a screw-capped test tube (15 \( \times \) 125 mm) and diluted with 0.5 mL of 0.1 M sodium acetate buffer (pH 3.6); 0.25 mL of kerosene was added, the tube was capped, and the mixture was shaken for 2 min at room temperature (around 28 \(^{\circ}\)C). The resulting uniform emulsion was allowed to sit for 10, 20, 30, 40 and 50 min, after which its absorbance (A) was measured at 540 nm with a UV-vis spectrophotometer (20 Genesis, BR). One unit (U) of emulsification activity was defined as that amount of emulsifier that affected an emulsion with A of 1.0 at 540 nm.

2.6.5. Surface tension determination

Surface tension was determined by a Tensiometer (Kruss K6) at 30 \(^{\circ}\)C, according to the De Nöuy ring method [15]. The surface tension determination was replicated at least three times and it was performed using cell-free supernatants.

2.6.6. Surfactin extraction

Surfactin extraction was performed according to the literature [11], with slight modifications: cells were separated by centrifugation at 10,000 \( \times \) g for 20 min at 15 \(^{\circ}\)C. The pH of 20 mL of cell-free supernatant was adjusted to 2.0 by adding 3 M HCl. The resultant solution was maintained at rest for 12 h to allow surfactin to settle. The precipitate was collected by centrifugation at 10,000 \( \times \) g for 15 min at 15 \(^{\circ}\)C, and the crude surfactin was obtained. For further purification, the crude surfactin was dissolved in 8.0 mL of deionized water and it was extracted three times by using an equal volume of dichloromethane (Vetec, São Paulo, Brazil). The solvent layer was harvested and evaporated at room temperature (around 28 \(^{\circ}\)C). The resulting brown-colored paste was dissolved in 2.0 mL methanol, achieving a solution containing a semi-purified surfactin.

2.6.7. Determination of surfactin concentration

Surfactin concentration was measured by HPLC using a Waters high-performance-liquid chromatographer equipped with a UV detector (Model 2487, Waters), at 205 nm, and a Symmetry C18 column (150 \( \times \) 4.6 mm, 5 \( \mu \)m, Waters, Ireland). The mobile phase consisted of 20% v/v TFA (3.8 mM) and 80% v/v acetonitrile. The elution rate was 1 mL/min at 30 \(^{\circ}\)C and the sample size was 20 \( \mu \)L. The identity of the semi-purified surfactin was obtained by using commercially available 95% pure surfactin (Sigma–Aldrich) as the authentic compound [16].

2.6.8. Determination of critical micelle concentration (CMC)

Different concentrations of the produced surfactin were obtained by performing several dilutions of cell-free fermented medium, after 48 h of fermentation [17]. Surface tension of the resulting solutions was measured at room temperature, as described above. The CMC was determined by plotting the surface tensions as a function of surfactin concentration and it was found from the intercept of two straight lines extrapolated from the concentration-dependent and concentration-independent sections [18].

2.6.9. Vibrational spectroscopy in the infrared region – FT-IR

The dried biomaterial was milled with KBr to form a very fine powder. This powder was then compressed into a thin pellet which could be analyzed by FT-IR spectra measurement, carried out by using a spectrometer model FT-IR 2000-ABB, with a spectral window from 400 to 4000 cm\(^{-1}\).

2.7. Calculation of fermentation parameters

Substrate conversion was calculated according to Fogler [19], see Eq. (2):

\[ \Delta S (\%) = \frac{S_0 - S}{S_0} \times 100 \]  

(2)

where \( S_0 \) is the initial substrate concentration (glucose plus fructose) and S the substrate concentration (glucose plus fructose) in the samples at each time interval.

The volumetric productivity (\( P_p \) and \( P_x \)) was calculated as the ratio of maximum biosurfactant (\( \text{P}_{\text{max}} \), mg/L) or cell concentration (\( \text{X}_{\text{max}}, \text{g/L} \)) to the fermentation time when the maximum concentration was achieved (\( t_{\text{max}} \) or \( t_{\text{Xmax}} \), respectively, h):

\[ P_p = \frac{P_{\text{max}}}{t_{\text{max}}} \]  

(3)

\[ P_x = \frac{X_{\text{max}}}{t_{\text{Xmax}}} \]  

(4)

The yield of biosurfactant on cell mass (\( Y_{P/X} \), g/g) was defined as:

\[ Y_{P/X} = \frac{P_f - P_0}{X_f - X_0} \]  

(5)

where \( P_0 \) and \( X_0 \) are the initial product and cell concentration, while \( P_f \) and \( X_f \) product and cell concentration in the samples at each time interval.

3. Results and discussions

3.1. Evaluation of cashew apple juice supplementation for optimal production of biosurfactant by Bacillus subtilis LAM005 in batch cultivation

In a previous work, the viability of biosurfactant production by \( B. \) \( \text{subtilis} \) LAM005 grown on a mineral medium formulated using clarified cashew apple juice and several nutrients was demonstrated [11]. Since the type of medium and growth conditions can influence the type and yield of the biosurfactant [20], in this work, the influence of juice supplementation on biosurfactant production was investigated, aiming at reducing costs of production. Fig. 1 shows the profile of substrate conversion, cell concentration, surfactin production by \( B. \) \( \text{subtilis} \) LAM005 and surface tension of the fermented broth free of cells after 30 h of fermentation using five different culture media, see Table 1.

It can be observed in Fig. 1A and B that the strain was able to grow and produce biosurfactant in all culture medium evaluated. The amount of surfactin varied from almost 35 mg/L to more than 175 mg/L, depending on the medium used. Furthermore, surface tensions (Fig. 1B) of the cell-free fermented broths were reduced from 53–54 dyne/cm to 29–30 dyne/cm, except for media D and E (around 36 dyne/cm). According to the literature [11,15,21], the effectiveness of a surfactant is determined by its ability in reducing surface tension of water below 35 dyne/cm. Therefore, the reduction of surface tension to those levels indicates the production of surface active compounds by \( B. \) \( \text{subtilis} \) LAM005. Fig. 1B also indicates a correlation between surfactin concentration and surface tension of the fermented broth free of cells, since an expressive decrease (below 30 dyne/cm) in surface tension is observed only
for media A–C, when more than 120 mg/L of biosurfactant concentration was achieved. It is worthy of note that regardless of the surfactant concentration, a further decrease in the surface tension will not be observed once the critical micelle concentration (CMC) has been reached [22]. Therefore, even though surfactin concentration in medium A is higher than in media B and C, the surface tension value in these samples is almost the same probably because the CMC has been achieved.

Since the highest surfactin production (higher than 140 mg/L) with the best tensioactive characteristics (decrease in surface tension to less than 30 dyne/cm) was achieved when media A and B were used, they were selected for further studies.

3.2. Effect of incubation time on growth and biosurfactant production by Bacillus subtilis LAM1005

The influence of incubation time on biosurfactant production and surface activities of culture supernatants was investigated. The profiles of substrate consumption, surfactin production and cell growth are pictured in Fig. 2A and B, respectively, when B. subtilis LAM1005 was cultivated in media A and B.

A plot of Ln(cell concentration) versus time (data not shown) allowed to observe an intense cell growth up to 48 h or 24 h of fermentation, for media A and B, respectively, when stationary phase was achieved. An experimental biosurfactant production (Y/μg) of 0.052 g surfactin/g cells (24 h) and 0.052 g surfactin/g cells (8 h) were obtained, respectively, for media A and B. The highest surfactin concentration (219.99 ± 0.4 mg/L) however, was achieved when B. subtilis LAM1005 was cultivated in medium B (Fig. 2B). Other authors [11], who studied the production of surfactin by the same microorganism using mineral media and clarified cashew apple juice (MM-CAJ), achieved a surfactin concentration around 125 mg/L, which is lower than the result obtained in this work.

The volumetric production rate (Y/P (g/Lh)) of surfactin, however, was higher for medium A (P = 7.3 mg/Lh) when compared to results in medium B (P = 4.2 mg/Lh). When B. subtilis LAM1005 was cultivated in medium A (Fig. 2A), maximum production of biosurfactant (around 177.93 ± 0.1 mg/L) was observed between 24 h and 48 h. However, the pH of the fermented broth reached values below 5.0 after 48 h (data not shown), which may have caused surfactin precipitation, underestimating surfactin concentration in the supernatant. Several authors [4,23–25], observed that surfactin precipitates at acidic pH values (≤5). Wei et al. [26], working with B. subtilis ATCC 21332 in iron-enriched culture, observed that the acidification of the broth caused surfactin precipitation resulting in the disappearance of soluble surfactin at pH lower than 5.0, whereas the insoluble surfactin was dissolved completely when the pH was increased back to pH 6.0 by adding NaOH.

Although high concentrations of glucose and fructose were still present in the culture medium (Fig. 2), an interruption of cellular growth and biosurfactant formation occurred. At the end of the process (72 h), total sugar (glucose and fructose) conversion was, respectively, 30.2% and 32.0% for media A and B. One possible explanation is a limitation on the nitrogen source needed for bacterial metabolism. Usually the carbon to nitrogen ratio is important to the product yield of bioactive molecules, which may have limited the process. Moreover, the end of the fermentation process may be associated with the formation of toxic by-products accumulation in the medium [27].

Based on the results of surfactin concentration, and considering that the cost of production of medium B is higher than the cost of medium A, which may be an obstacle on the way of industrial application, medium A was selected for further studies.

3.3. Kinetics of biosurfactant production by Bacillus subtilis LAM1005 considering the effect of carbon source concentration

To investigate the kinetic behavior of B. subtilis LAM1005 growth and biosurfactant production in batch cultivation, CCAJ was diluted with water, to achieve different initial concentrations of total reducing sugars (glucose and fructose), and supplemented with ammonium sulfate (1.0 g/L). Sugar concentration varied from 3 g/L to 96 g/L (undiluted juice). Fig. 3 shows the experimental results of cell growth, surfactin production, substrate consumption and pH of the fermented broth along time.

Fig. 4A shows a sigmoidal growth trend for the B. subtilis LAM1005 cells, in which the exponential growth phase and the stationary phase can be observed. A plot of Ln(cell concentration) versus time (data not shown) allowed observing that, when more than 48.96 g/L of initial sugar concentration was used, the cells entered an exponential growth phase before 10 h and continued up to 48 h. Moreover, a stationary phase was reached, which started at 48 h and lasted until 72 h. When more than 12.71 g/L of initial sugar concentration was used, the stationary phase was reached at 48 h. Maximum cell concentration and volumetric biomass
productivity increased with increasing sugar concentrations up to 65.04 g/L, when it remained almost constant, see Table 2.

Similar to cell concentration, maximum product (surfactin) concentration was also affected by the increase in sugar concentration in the culture medium, Fig. 3B and Table 2. Product concentration increased with increasing sugar concentration up to 65.04 g/L. When sugar concentration was enhanced to 96.1 g/L, biosurfactant production was lower. The highest concentration of surfactin (319.3 ± 0.1 mg/L) was achieved after 72 h of incubation of Bacillus subtilis LAMI005 on CCAJ, at an initial sugar concentration of 65.04 g/L, supplemented with ammonium sulfate (1.0 g/L). Other authors [28], who studied the production of surfactin by Bacillus subtilis ATCC 21332 using glucose as carbon source, achieved maximum surfactin concentrations in various nutritional conditions ranging

Table 2

<table>
<thead>
<tr>
<th>Run</th>
<th>( S_0 ) (g/L)</th>
<th>pH</th>
<th>( \Delta S ) (%)</th>
<th>( Y_{XX} ) (g/g)</th>
<th>( X_{max} ) (g/L)</th>
<th>( P_{max} ) (mg/L)</th>
<th>( P_X ) (g/L.h)</th>
<th>( P_Y ) (mg/L.h)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>12.71 ± 0.05</td>
<td>3.9 ± 0.56</td>
<td>38.6 ± 0.06</td>
<td>0.0148</td>
<td>2.0 ± 0.16</td>
<td>30.7</td>
<td>0.09</td>
<td>1.3</td>
</tr>
<tr>
<td>2</td>
<td>48.96 ± 0.09</td>
<td>4.0 ± 0.16</td>
<td>30.2 ± 4.16</td>
<td>0.0523</td>
<td>7.5 ± 0.18</td>
<td>147.9</td>
<td>0.14</td>
<td>3.7</td>
</tr>
<tr>
<td>3</td>
<td>65.04 ± 0.29</td>
<td>5.0 ± 0.62</td>
<td>34.3 ± 0.33</td>
<td>0.0450</td>
<td>8.6 ± 0.94</td>
<td>319.3</td>
<td>0.20</td>
<td>4.4</td>
</tr>
<tr>
<td>4</td>
<td>96.10 ± 1.42</td>
<td>6.8 ± 0.01</td>
<td>28.7 ± 1.34</td>
<td>0.0354</td>
<td>8.4 ± 0.12</td>
<td>214.2</td>
<td>0.17</td>
<td>4.0</td>
</tr>
</tbody>
</table>

\( ^a \) After 72 h of incubation.  
\( ^b \) Calculated for the growth phase.
from 31.2 to 439.0 mg/L. Volumetric biosurfactant productivity \( P_{P} \) was also affected by the initial substrate concentration in the culture medium. \( P_{P} \) increased with increasing sugar concentrations up to 65.04 g/L, when it remained almost constant, see Table 2.

The production of biosurfactant can alternatively be evaluated through the yield of biosurfactant on cell mass \( (Y_{P/X}) \), a volume-independent parameter very useful in scale up [10]. It can be observed in Table 2 that there is an optimal initial substrate concentration to this parameter, \( S_0 = 48.96 \). A decrease or increase in \( S_0 \) was expressed in a lower \( Y_{P/X} \). Other authors [28], working with \( B. \ subtilis \) ATCC 21332, obtained a \( Y_{P/X} \) ranging from 0.0068 to 0.075 g/g, depending on the nutritional condition evaluated.

According to some authors [3,16,29,30], glucose and fructose, which are present in cashew apple juice, are suitable substrates for the synthesis of biosurfactant. However, low substrate conversion (less than 40%), see Table 2, was achieved in all assays, which indicated that carbon limitation did not occur. Since an increase in cell concentration, together with higher product formation, is observed when sugar concentration is increased, it is possible that another nutrient present in the juice, for instance, amino-acids, favors biosurfactant production. Other authors [28], who used 30 g/L glucose as carbon source and 1.0 g/L of ammonium nitrate as nitrogen source under aerobic and nitrogen limited condition with \( B. \ subtilis \) ATCC 21332, also observed large amounts of residual glucose at the end of the assay. It is also important to notice that expressive substrate consumption, see Fig. 3C, started between 8 and 24 h of incubation, which may indicate a lag phase have occurred before this period.

After 72 h of cultivation, the acidification of culture medium was observed; except for run 4 \((S_0 = 96.1 \text{ g/L})\), see Table 2 and Fig. 3D. It can also be observed that media prepared with small amounts of juice became more acidic. This may be explained by the buffer effect provided by protein constituents present in cashew apple juice [9]. Another hypothesis for the fast pH decrease is the change from aerobic to anaerobic respiration. \( B. \ subtilis \) grows in the absence of oxygen using nitrate ammonification and various fermentation processes. Lactate, acetate, acetoin, ethanol, and succinate are the
main fermentation products. The change to anaerobic respiration could be induced by the accumulation of acidic compounds such as pyruvate and acetate from fermentation [31].

Some authors suggest [24,32] that biosurfactant production by B. subtilis is associated with growth, while other authors observed that the biosurfactant production occurs mainly at the end of the exponential growth phase [33] or at the stationary phase of biomass growth [34]. Several factors are responsible for this behavior, among which we should mention the type of organism being used and the culture medium in which it is grown. Therefore the analysis of the profiles of ln(X/X_0) versus time (t) and product concentration (P) versus time, during biosurfactant production by B. subtilis LAMI005 on CCAJ, supplemented with ammonium sulfate at a concentration of 1.0 g/L, were compared in order to distinguish between the three broad kinetic groups proposed by Elmer and Gaden [35]. Results are shown in Fig. 4, for S_0 = 65.04.1 g/L. It can be observed that ln(X/X_0) × t and P × t have similar profiles, in other words, there is a connection between the curves. Production kinetics is parallel to the biomass kinetics to a large extent during logarithmic growth. Similar profiles were obtained for the other initial substrate concentration studied (data not shown). Therefore, in the conditions assayed here, surfactin production by B. subtilis LAMI005 is associated with growth.

3.4. Evaluation of the surface activity of the biosurfactant produced by B. subtilis LAMI005 cultivated in CCAJ supplemented with ammonium sulfate

The stability of oil/water emulsions is also widely used as an indicator of surface activity, although the ability of a molecule to form a stable emulsion is not associated with surface tension reduction [36]. The rate of emulsification determines the ability of the biosurfactant to form emulsions, and the ability to stabilize emulsions is also a parameter used to evaluate the surface activity of surfactants. There are some examples in the literature of biosurfactant that both lowered surface tension and stabilized emulsions. On the other hand, some Bacillus species produce a type of emulsifier that may not cause an appreciable reduction of the surface tension of water but it is able to form an excellent emulsion with kerosene, for instance [12]. Therefore, the surface activity of biosurfactant produced by B. subtilis LAMI005 cultivated in CCAJ supplemented with ammonium sulfate, at different initial substrate concentrations, was characterized by measuring the surface tension, emulsifying activity and emulsification index of the fermented broth free of cells.

Table 3 shows the emulsification index measured at the fermentation time where the highest biosurfactant concentration was achieved, which varied depending on the initial substrate concentration used. The biosurfactant produced by LAMI005 showed high emulsification index (IE_{24} > 50%) on kerosene and soybean oil, but not against gasoline. Most microbial surfactants are substrate specific, solubilizing or emulsifying different hydrocarbons at different rates [37]. Best results for emulsification index (IE_{24}) were obtained by using kerosene (67%), followed by soybean oil (64%). Similar results were obtained by other authors [9], after 72 h of cultivation, 65% of kerosene emulsification was obtained, indicating that this biosurfactant has an emulsifying activity.

The ability to stabilize an emulsion is an indication that the microorganism is producing biosurfactant [38]. Results of emulsifying capacity and stability can be seen in Fig. 5. The biosurfactant
produced by *B. subtilis* LAMI005 showed an emulsification activity higher than 1.0 U, except when *S*₀ = 12.71 g/L was used. It is worth noticing that in this condition, low amounts (concentration) of biosurfactant was produced. Higher emulsifying capacity is also observed on the sample collected after 48 h of inoculation. The emulsion stability is always very high and independent of the initial substrate concentration on the culture medium. Results obtained in this work are similar to those described for the synthetic commercial surfactants tested by Amaral et al. [39]. They were also similar to those described to other biosurfactant from *Yarrowia lipolytica* [39], *C. lipolytica* IA [40] and Nocardia sp. L-417 [41].

3.5. Minimum surface tension and critical micelle concentration considering the effect of carbon source concentration

It is important to distinguish between an effective biosurfactant and an efficient biosurfactant. Effectiveness is measured by the minimum value to which the surface tension can be reduced, whereas efficiency is measured by the biosurfactant concentration required to produce a significant reduction in the surface tension of water. The latter can be determined from the CMC of the biosurfactant [18]. Moreover, the ability to reduce surface tension below 35 dyne/cm is one of the criteria used to select biosurfactant-producing microorganisms [1]. Several factors influence the type and amount of surfactant, among which we highlight the growing conditions and nutrient availability in the medium [42,43]. In this work, the effect of carbon source concentration on biosurfactant effectiveness and efficiency was investigated. Fig. 6 shows the results of surface tension of the cell-free supernatant along time for the different experimental conditions evaluated. It can be observed that, in all assays (runs 1–4) *B. subtilis* LAMI005 produced biosurfactant, since surface tension values below 35 dyne/cm were detected.

It is possible to observe in Fig. 6 that as surfactant concentration increases in the culture medium (see Fig. 3B), lower values of surface tension are achieved up to a minimum value (30 dyne/cm). In some cases, when using 65.04 and 96.1 g/L of initial substrate concentration, the surface tension remained almost unaltered from 8/24 h until the end of cultivation. After this time of cultivation, although surfactin concentration continued to grow, no further reduction was observed in the surface tension of the free-cell broth. Similar behavior was observed by other authors [44] and they suggested that the biosurfactant concentration in the broth probably reached or exceeded its CMC at this cultivation time. According to the literature [18], as surfactant concentration increases the surface tension of the surfactant solution decreases up to a certain value and then becomes almost constant due to the interface saturation with the surfactant molecules. Barros et al. [23], working with *B. subtilis* LBS, grown in cassava wastewater, report that the biosurfactant tested exhibited excellent surface activity, reducing the tension of water from 72.31 to 27.01 dyne/cm. Queiroga et al. [45] using *B. subtilis* for the production of biosurfactant in the presence of oil, reported a reduction in surface tension of the fermented broth from 53 dyne/cm to 25.7 dyne/cm. Gouveia et al. [46], working with 13 biosurfactant-producing strains, and using glycerol and glucose as carbon source, observed a reduction in surface tension of the medium from 58 dyne/cm to 30 dyne/cm. In this work, the produced surfactin showed to be a highly effective biosurfactant since its crude solution could lower the surface tension of the fermented broth to 30 dyne/cm. However, it was documented that surfactin can lower the surface tension to even lower values, reaching 27 dyne/cm [1]. This contradiction may be attributed to the low purity of the surfactin preparation, since it was not extracted from the culture medium.

By definition, the CMC is the surfactant concentration at which an abrupt increase in surface tension is observed. Regardless of the surfactant concentration, a further decrease in the surface tension will not be observed once the CMC has been reached [22]. Zhang and Miller [47] reported that the concentration of biosurfactant required to reach the CMC is typically between 1 and 200 mg/L, while the interfacial tension (oil/water) is around 1 and 30 dyne/cm [48]. Table 4 shows the critical micelle concentration (CMC) of surfactin produced by *B. subtilis* LAMI005 at this work, as well as SDS and several biosurfactant isolated from different *B. subtilis* strains, including a standard sample (surfactin from Sigma-Aldrich).

It can be observed in Table 4 that surface active compounds can reduce the surface tension of water to values around 27–37 dyne/cm and their CMCs range from 15 to 180 mg/L. Purified surfactin (standard) is even more efficient since its CMC could reach 7.8 mg/L, see Table 4. Variations in the values of CMC [13, 22, and 17 mg/L] for surfactin have been described by other authors [51–53]. The literature [4,52] reports that these variations are observed depending on the nature of the solvent used to dissolve

### Table 3

Emulsification index (IE₂₄) of the biosurfactant produced by *Bacillus subtilis* LAMI005 grown in clarified cashew apple juice, supplemented with ammonium sulfate (1.0 g/L) at 30 °C and 180 rpm, using different initial concentrations of substrate (*S*₀). Experiments were performed in duplicate and the results represent means ± standard deviations of the two independent experiments.

<table>
<thead>
<tr>
<th>Run</th>
<th><em>S</em>₀ (g/L)</th>
<th>Fermentation time (h)</th>
<th>IE₂₄ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Soybean oil</td>
</tr>
<tr>
<td>1</td>
<td>12.71 ± 0.05</td>
<td>24</td>
<td>0.0</td>
</tr>
<tr>
<td>2</td>
<td>48.96 ± 0.09</td>
<td>48</td>
<td>56 ± 0.12</td>
</tr>
<tr>
<td>3</td>
<td>65.04 ± 0.29</td>
<td>72</td>
<td>53 ± 0.06</td>
</tr>
<tr>
<td>4</td>
<td>96.10 ± 1.42</td>
<td>72</td>
<td>64 ± 0.00</td>
</tr>
</tbody>
</table>

![Fig. 6. Surface tension of the fermented broth free of cells after 72 h of fermentation of clarified cashew apple juice (CCA) + ammonium sulfate (1.0 g/L) with different initial substrate concentrations: ( ) 12.71 g/L, ( ) 48.96 g/L, ( ) 65.04 g/L, ( ) 96.10 g/L (undiluted juice). Error bars illustrate experimental errors (standard deviations), calculated from two independent experiments.](Image 89x147 to 331x339)
Table 4
Minimal surface tensions and critical micelle concentration (CMC) obtained for several surface active compounds. Experiments were performed in duplicate and the results represent means ± standard deviations of the two independent experiments.

<table>
<thead>
<tr>
<th>Surfactant</th>
<th>Surface tension (dyn/cm)</th>
<th>CMC (mg/L)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Run 1 – S_1 = 12.71 g/L</td>
<td>34.7 ± 0.0</td>
<td>10.24</td>
<td>This work</td>
</tr>
<tr>
<td>Run 2 – S_2 = 48.96 g/L</td>
<td>30.0 ± 0.0</td>
<td>16.90</td>
<td>This work</td>
</tr>
<tr>
<td>Run 3 – S_3 = 65.04 g/L</td>
<td>30.6 ± 0.5</td>
<td>63.00</td>
<td>This work</td>
</tr>
<tr>
<td>Run 4 – S_4 = 96.10 g/L</td>
<td>30.0 ± 0.0</td>
<td>21.41</td>
<td>This work</td>
</tr>
<tr>
<td>SDS (synthetic)</td>
<td>37.0</td>
<td>2.888</td>
<td>[49]</td>
</tr>
<tr>
<td>Surfactin from Bacillus subtilis isolate bs5</td>
<td>42.5</td>
<td>15.6</td>
<td>[18]</td>
</tr>
<tr>
<td>Surfactin from Bacillus subtilis</td>
<td>27</td>
<td>25.0</td>
<td>[32]</td>
</tr>
<tr>
<td>Standard surfactin (Sigma–Aldrich)</td>
<td>7.8–20.7^a</td>
<td></td>
<td>[50]</td>
</tr>
</tbody>
</table>

a CMC = 7.5–20 μmol/L and molecular weight = 1036.34 g mol [50].

Fig. 7. A comparison of vibrational spectroscopy in the infrared region – FT-IR, spectograms of standard surfactin (dashed line) and semi-purified extracellular biosurfactant (solid line) produced by B. subtilis LAM1005 grown in clarified cashew apple juice (CCAJ) supplemented with ammonium sulfate (1 g/L) at 30 °C, 180 rpm and S_0 = 65.04 g/L.

surfactin as well as the purity of surfactin preparation. By comparison, the results obtained in this work are in agreement with those obtained from the literature, which shows the potential of using CCAJ as a carbon source for surfactin production by B. subtilis strains.

3.6. Chemical structure of the surfactant produced by B. subtilis grown in clarified cashew apple juice

Fig. 7 shows the infrared spectrum of the B. subtilis LAM1005 biosurfactant and the spectrum of a standard sample of surfactin from B. subtilis (Sigma–Aldrich, 95% purity). In both spectra it is possible to observe bands characteristic of peptides (wave number 3430: NH, wave number 1655: CO, and wave number 1534: CN) and aliphatic chains (wave number 3000–2800, CH2 and CH3), indicating that this compound is a lipopeptide. A band corresponding to an ester carbonyl group (wave number 1730: CO) is also observed. Similar results were obtained by other authors [54] when determining the chemical structure of the surfactant produced by Bacillus licheniformis. No significant difference in IR spectra of the biosurfactant produced in this work or the standard sample is observed.

4. Conclusions

B. subtilis LAM1005 was capable to grow and to produce biosurfactant in an alternative culture media, prepared by using clarified cashew apple juice, an agroindustrial by-product of the cashew industry, supplemented with ammonium sulfate. Since economic considerations are one of the main problems concerning the expansion of the biosurfactant market, CCAJ appears as an alternative water soluble low cost medium. Based on the literature [7], this result is interesting once water-soluble substrates are cheaper than hydrocarbons and are preferred because single-phase fermentation is simpler than biphasic fermentation. Analyses of the culture supernatants along cultivation time showed that surfactin production was influenced by the amount of CCAJ used to prepare the culture medium, reaching a maximum concentration of 319.3 mg/L. Furthermore the emulsification index (IE24) value of the biosurfactant was found to be 65% demonstrating its capacity to emulsified kerosene and soybean oil. The biochemical analysis of the semi-purified biosurfactant indicates that surfactin was produced by B. subtilis LAM1005 under the conditions assayed in this work.

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References

biochemical process


