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Biosurfactant Production by *Bacillus subtilis* Using Cassava-Processing Effluent

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Abstract

A cassava flour-processing effluent (*manipueira*) was evaluated as a substrate for surfactant production by two *Bacillus subtilis* strains. *B. subtilis* ATCC 21332 reduced the surface tension of the medium to 25.9 mN/m, producing a crude biosurfactant concentration of 2.2 g/L. The wild-type strain, *B. subtilis* LB5a, reduced the surface tension of the medium to 26.6 mN/m, giving a crude biosurfactant concentration of 3.0 g/L. A decrease in surfactant concentration observed for *B. subtilis* ATCC 21332 seemed to be related to an increase in protease activity. The biosurfactant produced on cassava effluent medium by *B. subtilis* LB5a was similar to surfactin.

Index Entries: Biosurfactant; *Bacillus subtilis*; cassava effluent; surfactin; protease.

Introduction

Naturally occurring surface-active compounds derived from microorganisms have gained attention in the past few decades because of their biodegradability, low toxicity, ecologic acceptability, and ability to be produced from renewable and cheaper substrates *(1,2)*. Biosurfactants find applications in the cosmetic, pharmaceutical, and food industries as emulsifiers, humectants, dispersants, and detergents *(3,4)*. Moreover, they are suited for environmental applications such as bioremediation, dispersion of oil spills, and waste treatment *(5)*. Among the many classes of biosurfactants, lipopeptides are of great interest because of their high surface activities and therapeutic potential *(6)*. The lipopeptide surfactin, produced by *B. subtilis* strains, is one of the most powerful biosurfactants discovered so far *(7)*. In addition to its exceptional surfactant activity, surfactin also shows numerous biologic properties such as antimicrobial *(8)*, antiviral *(9)*, and antitumoral *(10)*.

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Production cost is considered the bottleneck of many biotechnological processes. The success of biosurfactant production depends on the development of cheaper processes and the use of low-cost raw materials, which account for 10–30% of the overall cost *(11)*. Molasses *(12)*, peat hydrolysate *(13)*, and potato process effluents *(14)* are examples of alternative substrates that have been suggested for biosurfactant production by *B. subtilis*.

Previous research showed that a cassava flour wastewater, also known in Brazil as *manipueira*, could be explored as an alternative substrate for surfactant production by *Bacillus* sp. *(15)*. In the present work, the production of biosurfactant by two *B. subtilis* strains using a cassava effluent medium was investigated.

Materials and Methods

Microorganisms

B. subtilis ATCC 21332, a known surfactin-producing strain, and the wild-type *B. subtilis* LB5a, selected based on previous work *(16)*, were maintained on nutrient agar (Difco) slants at 4°C.

Preparation of Substrate

Cassava effluent obtained from the manufacturing of cassava flour was collected and stored at –18°C until needed. The medium was prepared by heating the waste until boiling for solids removal. After cooling, the substrate was centrifuged at 8000*g* for 20 min (Beckman model J2-21). The supernatant was distributed into flasks and sterilized in an autoclave at 1 atm, 121°C for 15 min. The natural pH of the medium was 5.9 and was not adjusted.

Inoculum and Culture Conditions

The bacterial strains were streaked on a nutrient agar slant and incubated for 24 h at 30°C. Two loops of culture were inoculated in 20 mL of nutrient broth (Difco) in a 50-mL Erlenmeyer flask and incubated on a rotary shaker (New Brunswick) at 150 rpm, 30°C for 8–12 h until the cell numbers reached 10^8 CFU/mL. A 1-mL aliquot of inoculum was transferred to 15 mL of cassava effluent medium contained in a 50-mL Erlenmeyer flask and incubated at 30°C, 150 rpm on a rotary shaker (New Brunswick). Samples were taken at time-defined intervals and analyzed. The experiments were carried out in three independent replicates.

Analytical Measurements

Viable cell numbers were determined by serial dilutions of the samples, and the viable counts were performed by a spread-plate technique using nutrient agar plates incubated at 30°C for 18 h. Total carbohydrates were estimated using a phenol-sulfuric assay *(17)*.

Surface activity measurements were performed after centrifuging the cultures at 8000*g* for 20 min for cell removal. The supernatant was submitted to analysis of surface tension, critical micelle dilution (CMD), and reciprocal critical micelle concentration (CMC^{-1}). Surface tension was determined as mN/m with a Krüss Processor Tensiometer (model K12 T Krüss; Hamburg, Germany) using the plate method. CMD^{-1} and CMD^{-2} were determined by measuring the surface tension of 10X and 100X diluted broth in distilled water. The maximum SD allowed for the ST measurements was 0.20.

The CMC^{-1} was determined by measuring the surface tension of serially diluted broth samples as previously reported by Sheppard and Mulligan *(13)*.

Biosurfactant was isolated from cell-free broth by precipitation after adjusting the broth's pH to 2.0 using 6 *N* HCl and keeping it at 4°C overnight. The precipitate thus obtained was centrifuged at 8000*g* for 20 min, dried, and weighted. The dry weight of crude precipitate was related to the initial sample volume and the biosurfactant concentration (g/L) was calculated.

For infrared (IR) spectroscopy, the crude surfactant sample was partially purified using the methodology described by Makkar and Cameotra *(18)*. Fourier transform IR absorption spectra of standard surfactin (Sigma) and LB5a biosurfactant were obtained using a Perkin-Elmer spectrophotometer (Model Spectrum One). Samples were dissolved in chloroform, and a drop of solution was dispersed in an NaCl pellet and maintained at dry atmosphere until solvent evaporation. The spectra were generated from 500 to 4000 cm^{-1} with a 4-cm^{-1} resolution.

Protease activity measurement was carried out in cell-free broth samples using the method described by Obata et al. *(19)*. One unit of enzyme activity was defined as the amount of enzyme required to produce an increase in absorbance equal to 1.0 in 30 min *(20)*.

Results

The fermentation kinetics of *B. subtilis* LB5a and *B. subtilis* 21332 using cassava effluent (*manipueira*) as substrate are shown in Figs. 1 and 2. Carbohydrate consumption and cell growth were very similar for both strains. The main carbohydrates present in cassava waste are sucrose, glucose, and fructose, and all sugars were consumed simultaneously during cultivation; that is, no diauxic effect was observed (data not shown). The cultures reached stationary growth phase after 12 h of cultivation. The pH of the strain 21332 medium initially increased and subsequently decreased to values between 7.0 and 7.6 at later cultivation period, whereas strain LB5a showed an increase in pH of the medium that was maintained between 7.5 and 8.0.

A minimal surface tension of 25.9 m*N*/m was attained after 24 h for strain 21332, whereas for LB5a the lowest surface tension value of 26.6 m*N*/m

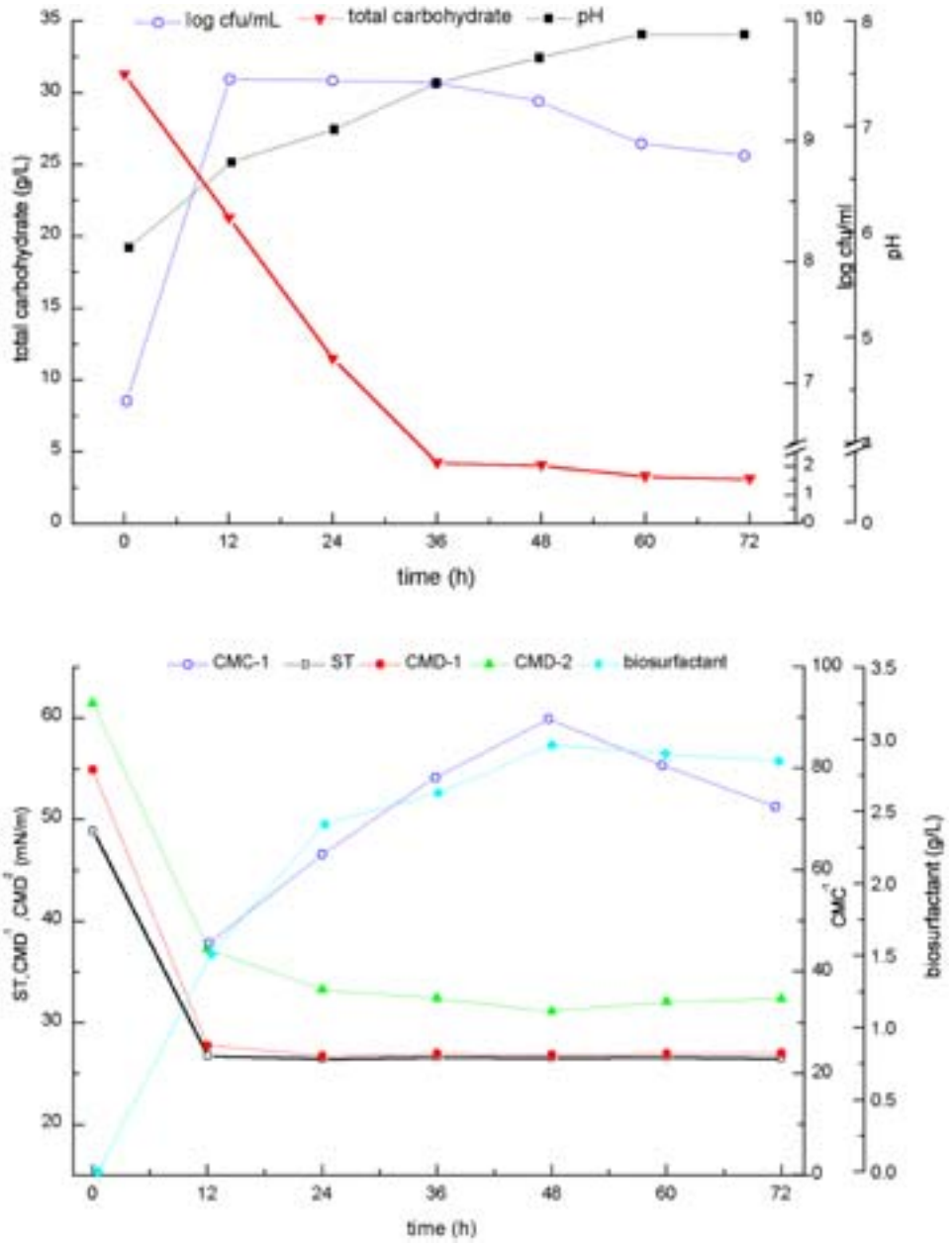

Fig. 1. Time course of biosurfactant production by *B. subtilis* LB5a.

was observed after 12 h. A biosurfactant concentration of 2.2 g/L was produced by *B. subtilis* 21332 after 36 h of cultivation, and *B. subtilis* LB5a reached a maximum biosurfactant concentration of 3.0 g/L after 48 h. The bulk of biosurfactant was produced after 12 h by the 21332 strain, whereas for LB5a 50% of surfactant biosynthesis occurred during the first 12 h of cultivation. The drastic drop in biosurfactant concentration observed for

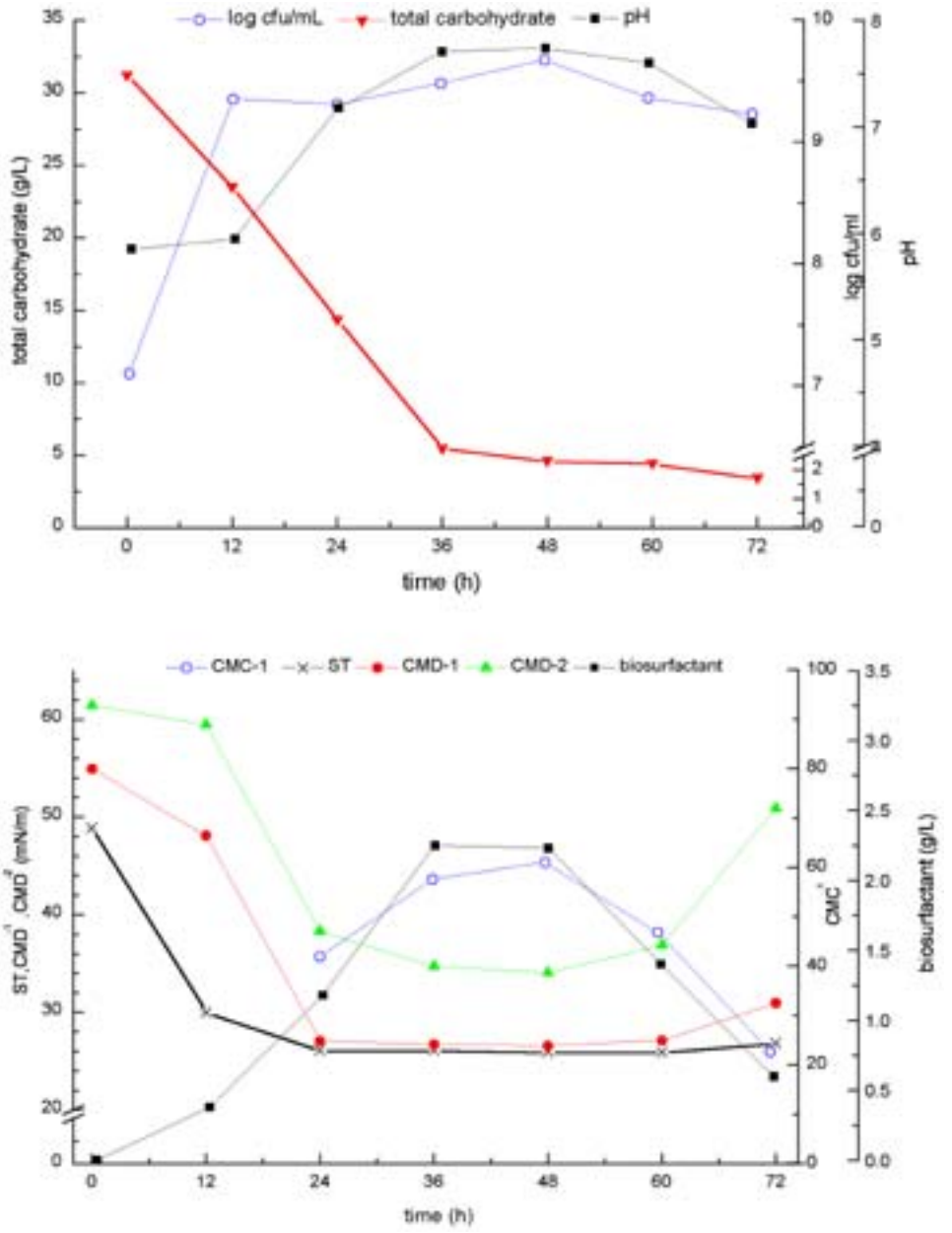

Fig. 2. Time course of biosurfactant production by *B. subtilis* ATCC 21332.

the 21332 strain was also accompanied by a change in surface activity parameters, i.e., an increase in surface tension and CMD and a decrease in CMC^{-1} (Fig. 2).

Figure 3 shows the correlation between surfactant concentration and protease production by strain 21332. The enzyme activity present in the culture medium showed an increase from 0.2 U/mL at 36 h to 1.03 U/mL

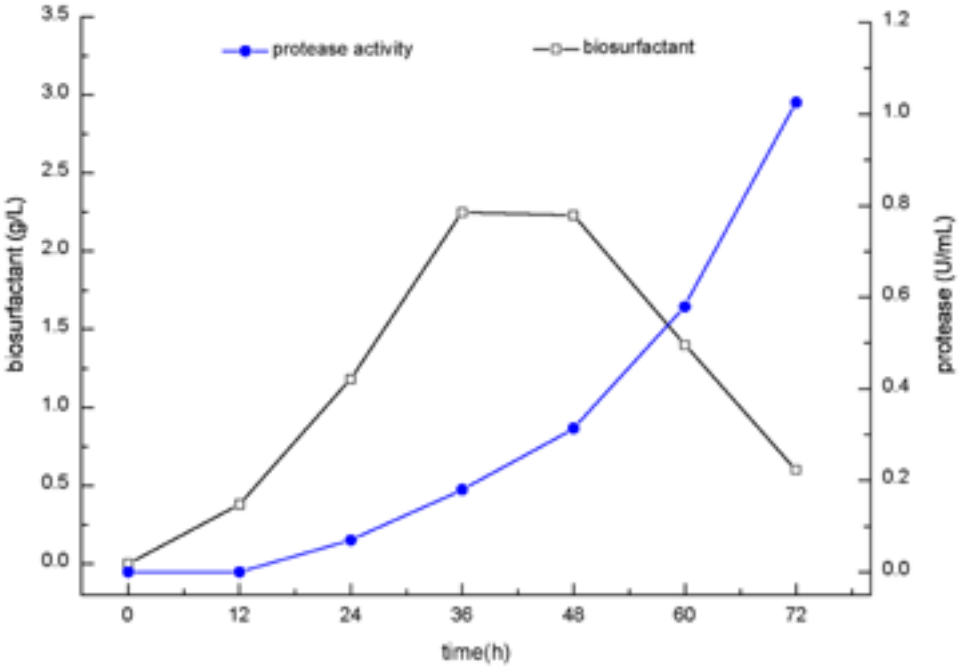

Fig. 3. Protease activity obtained during cultivation of ATCC 21332 on cassava effluent medium.

Table 1
Addition of Surfactant in Culture Broth From *B. subtilis* ATCC 21332

	Surface activity (mN/m)		
Sample[a]	Surface tension	CMD^{-1}	CMD^{-2}
Broth (control)	26.01	27.91	48.48
Broth + 20 µg of surfactant[b] (0 h)	25.91	26.60	33.01
Broth + 20 µg of surfactant[b] (24 h)	26.38	29.55	48.93

[a]Fifteen milliliters of *B. subtilis* 21332 broth after 72 h of fermentation.
[b]Crude surfactant from *B. subtilis* LB5a.

at 72 h (5.15-fold), and simultaneously the surfactant concentration showed a decrease from 2.2 to 0.6 g/L (73% decrease).

Table 1 shows the addition of crude surfactant from strain LB5a in 72-h cultivation broth of *B. subtilis* 21332. When surfactant was added, the surface activity measurements obviously decreased (at time zero), but after 24 h of cultivation at 30°C and 150 rpm, the surface tension and CMD values increased. The CMD^{-2} values increased from 33.01 to 48.93 mN/m after 24 h, reaching almost the same value of the initial control medium without addition of surfactant.

The IR spectra of commercial surfactin and the semipurified surfactant produced by LB5a on cassava effluent are shown in Fig. 4. The spectra

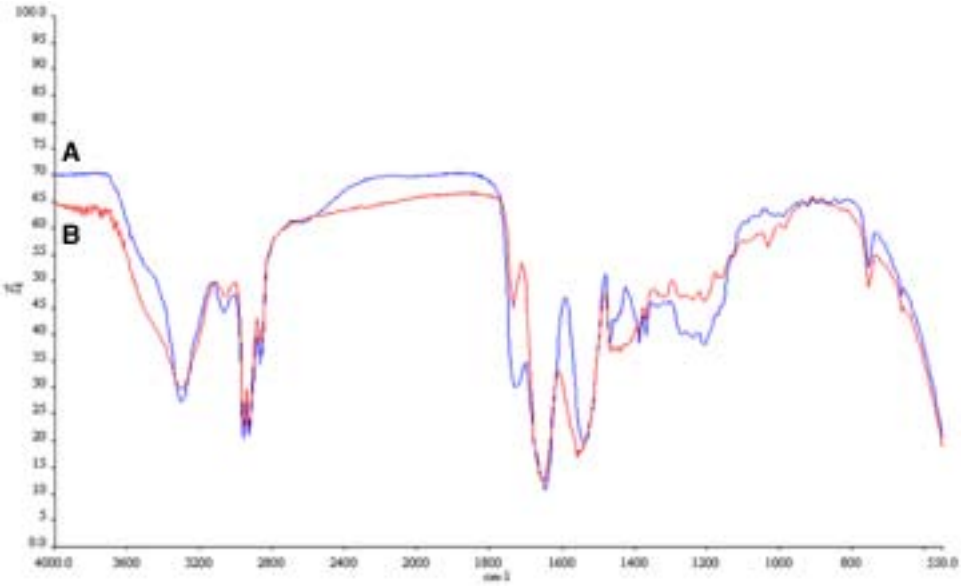

Fig. 4. IR spectra of standard surfactin **(A)** and surfactant produced by *B. subtilis* LB5a on cassava effluent medium **(B)**.

were very similar, indicating that the product obtained was a surfactin-like surfactant (lipopeptide). The spectrum of LB5a biosurfactant was not as sharp as that of standard surfactin because of the presence of impurities.

Discussion

The strains tested were capable of growing and producing biosurfactant on cassava effluent medium, although the surfactant biosynthesis had demonstrated some differences. *B. subtilis* LB5a accumulated surfactant during exponential and stationary growth phases as reported previously for other *Bacillus* strains *(6)*. For *B. subtilis* 21332, the bulk of surfactant was synthesized during stationary phase, indicating that surfactant production was not directly associated with cell growth. It has been reported that the onset of surfactin production by *B. subtilis* 21332 was in the mid-to-late log phase *(7,13)*, which we also observed here.

The minimum surface tensions were attained after 12 (LB5a) and 24 h (21332), meaning that, at these cultivation times, the surfactant concentrations present were in excess of their CMC, and a further increase in biosurfactant concentration did not decrease surface tension. The CMD values, mainly CMD^{-2}, are a more useful index because at this dilution, the surfactant is not above CMC and any change in surfactant concentration is reflected on CMD^{-2}. The CMC^{-1} is a direct indication of surface activity in the broth *(13)*: the higher the CMC^{-1}, the greater the surface activity (Figs. 1 and 2).

When media nutrients were depleted (in the late cultivation period), the surfactin concentration on culture medium of *B. subtilis* 21332 was generally constant and no further increase was detected *(21)*. This behavior was also observed for strain LB5a. The decrease in biosurfactant concentration demonstrated for *B. subtilis* 21332 growing on cassava waste suggests a product degradation. Wei and Chu *(22)* reported a drop in surfactin levels when iron was added to culture medium of *B. subtilis* 21332, but the decrease in surfactant concentration was owing to an acidification of culture broth that caused a product precipitation. In our case, the pH of the medium when the surfactant level decreased was about 7.0, and no precipitation was detected. The changes observed in the surface activity parameters support the idea that biosurfactant had been consumed or degraded.

It is known that *Bacillus* strains can produce proteases *(23)*. The increasing protease activity on culture medium of *B. subtilis* 21332 (Fig. 3) led us to conclude that the decrease in biosurfactant concentration could be owing to an enzymatic action. Grangemard et al. *(24)*, working with a bacterial endoprotease produced by *Staphylococcus aureus*, first reported the enzymatic hydrolysis of surfactin. The enzyme acted on the peptide moiety of surfactant, generating an open-chain lipopeptide.

The reason for the final product consumption could be the energetic needs of the bacterial cells. Enzymatic hydrolysis would provide some nutrients that could be used for cell maintenance. A severe decline in biosurfactant concentration was not observed for *B. subtilis* LB5a even though a protease activity of 5.3 U/mL was present (data not shown); this fact suggests that the protease of *B. subtilis* 21332 is probably different from the LB5a protease.

Considering the surface activity values in Table 1, it can be noted that the concentration of LB5a biosurfactant decreased on *B. subtilis* ATCC 21332 broth, suggesting an action of the enzyme produced by this microorganism on the surfactant molecule. *B. subtilis* 21332 seemed to be able to express a protease for its own product degradation (surfactin). One explanation for these facts could be that when surfactant concentration reaches certain levels, it causes a toxic effect on bacterial cells and the microorganism degrades the surfactant to avoid inhibition of growth or even cell death. Probably, *B. subtilis* 21332 cells are more sensitive to surfactant effects than *B. subtilis* LB5a.

Although *B. subtilis* cells are known to produce proteases, the enzymatic hydrolysis of surfactin in the culture medium was not reported. Our results could be related to the cassava effluent; that is, some component present on this medium stimulates the production of some specific protease by *B. subtilis* 21332. Another supposition is that the exhaustion of an essential nutrient in the *manipueira* medium induces the bacteria to produce an enzyme to hydrolyze surfactin and use it as a substrate. The protease produced by *B. subtilis* 21332 on cassava waste medium should be identified, purified, and characterized.

The increasing interest in the study of peptide synthetase systems would permit modifications on the peptide moiety of lipopeptides *(25)*. Moreover, some proteases act as catalysts to their reverse action—i.e., the formation of peptide bound *(26)*—and, therefore, an enzymatically driven amino acid substitution *(27)* can be developed. By combining the action of a protease that hydrolyzes the peptide moiety of lipopeptides with the action of a biosynthetic enzyme, it will be possible to substitute amino acids and consequently modify the properties and the biologic activity of these compounds. Proteases that act on lipopeptides, such as surfactin, would be useful tools for these special modifications.

The biosurfactant produced by *B. subtilis* LB5a on *manipueira* medium is similar to surfactin, and its structural and physicochemical characterization are under investigation.

Conclusion

Cassava-processing effluent (*manipueira*) proved to be a potentially useful substrate for biosurfactant production by *B. subtilis* LB5a. Utilization of this medium for surfactant production by *B. subtilis* ATCC 21332 is possible, but the cultivation time has to be considered to avoid loss of the final product.

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