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Production and properties of a surfactant obtained from *Bacillus subtilis* grown on cassava wastewater

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Abstract

The production and properties of a biosurfactant, synthesized by *Bacillus subtilis* LB5a strain, using cassava wastewater as substrate were investigated. The microorganism was able to grow and to produce surfactant on cassava waste, reducing the surface tension of medium to 26.6 mN/m and giving a crude surfactant concentration of 3.0 g/L after 48 h. The surface-active compound retained its properties during exposure to elevate temperatures (100 °C), high salinity (20% NaCl) and a wide range of pH values. The surfactant was capable of forming stable emulsions with various hydrocarbons. Preliminary chemical characterization revealed that the surfactant has a lipopeptide composition with a CMC value of about 33 mg/L. Cassava wastewater proved to be a suitable substrate for biosurfactant biosynthesis, providing not only bacterial growth and product accumulation but also a surfactant that has interesting and useful properties with potential for many industrial applications.
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Keywords: Biosurfactant; *Bacillus subtilis*; Cassava wastewater; Lipopeptide

1. Introduction

Biosurfactants are surface-active compounds produced by microorganisms. Most microbial surfactants are complex molecules, comprising different structures that include lipopeptides, glycolipids, polysaccharide-protein complex, fatty acids and phospholipids. In the past few decades, biosurfactants have gained attention because they exhibit some advantages such as biodegradability, low toxicity, ecological acceptability and ability to be produced from renewable and cheaper substrates (Desai and Banat, 1997; Rosenberg and Ron, 1999). The range of industrial applications of biosurfactants includes enhanced oil recovery, crude oil drilling, lubricants, bioremediation of pollutants, health care and food processing (Banat et al., 2000). Among the many classes of biosurfactants, lipopeptides from *Bacillus subtilis* are particularly interesting because of their high surface activity and therapeutic potential (Besson and Michel, 1992; Sandrin et al., 1990).

Although biosurfactants exhibit such important advantages, they have not been yet employed extensively in industry because of relatively high production costs. One possible strategy for reducing costs is the utilization of alternative substrates such agro industrial wastes (Mercade and Manresa, 1994). The main problem related to use of alternative substrates as culture medium is to find a waste with the right balance of nutrients that permits cell growth and product accumulation (Makkar and Cameotra, 1999). Molasses (Makkar and Cameotra, 1997b), peat hydrolysate (Sheppard and Mulligan, 1987) and potato process effluents (Fox and Bala, 2000) are examples of alternative substrates that have been suggested for biosurfactant production by

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B. subtilis. The establishment of waste-based medium for biosurfactant production also faces another problem, once the kind and the properties of final product are dependant on the composition of culture media (Besson and Michel, 1992).

Cassava wastewater is a carbohydrate rich residue generated at large amounts during the production of cassava flour, a very common ingredient used on Brazilian cookery. The major nutrients present on cassava waste are sugars and mineral salts. The disposable of this residue causes environmental problems due to its high organic load; however, it is a very attractive substrate for biotechnological processes. Previous work showed that this waste could be a potential substrate for biosurfactant production (Nitschke and Pastore, 2003). In this study, we report the production of biosurfactant using cassava wastewater by *B. subtilis* LB5a. The properties and initial chemical characterization of the product obtained are also presented.

Table 1
Composition of cassava wastewater utilized on this work

Components	Concentration
Total carbohydrates (g/L)	35.3 ± 1.52
Reducing sugars (g/L)	12.8 ± 0.57
No reducing sugars (g/L)	22.2 ± 0.35
Total nitrogen (g/L)	2.5 ± 0.17
Phosphorous (mg/L)	225.9 ± 0.34
Potassium (mg/L)	2665.1 ± 0.45
Calcium (mg/L)	272.5 ± 0.15
Magnesium (mg/L)	519.0 ± 0.09
Sulfur (mg/L)	104.0 ± 0.21
Iron (mg/L)	7.8 ± 0.03
Zinc (mg/L)	7.3 ± 0.08
Manganese (mg/L)	1.8 ± 0.05
Copper (mg/L)	0.6 ± 0.06
pH	5.9 ± 0.02
COD[a] (g O_2/L)	55.82 ± 0.25

[a] COD: chemical oxygen demand.

2. Methods

2.1. Microorganisms

B. subtilis LB5a from Biochemistry Laboratory Culture Collection (Nitschke and Pastore, 2003), was maintained on nutrient agar (Difco) slants at 4 °C.

2.2. Substrate preparation

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 to facilitate the removal of insoluble solid material. After cooling, the substrate was centrifuged at 8000*g* for 20 min (Beckman model J2-21). The supernatant was distributed in flasks and sterilized in autoclave at 1 atm, 121 °C for 15 min. Natural pH of the medium was 5.9 and was not adjusted. The same cassava wastewater batch was used for all experiments and its composition is summarized in Table 1.

2.3. Inoculum and culture conditions

The bacterial strains were streaked in 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 in a rotary shaker (New Brunswick) 150 rpm at 30 °C for 8–12 h until cell numbers reach 10^8 cfu/mL. An aliquot of 5 mL of inoculum was transferred to 75 mL of cassava effluent medium contained in a 250 mL Erlenmeyer flask and incubated at 30 °C, 150 rpm in a rotary shaker (New Brunswick). Samples were collected at time-defined intervals and submitted to analysis. The experiments were conducted in three independent replicates.

2.4. Analytical measurements

Viable cell numbers. Samples were submitted to serial dilutions and viable counts were performed by spread plate technique.

Carbohydrates. Total carbohydrates were estimated using the phenol–sulfuric assay (Daniels et al., 1994). The sugars present on cassava substrate (glucose, fructose, maltose, sucrose) were measured by HPLC using a Waters high-performance-liquid chromatography equipped with a refractive index detector and a YMC Pack Polyamine II column (4.6 × 250 mm). A mixture of acetonitrile:water (75:25) was used as solvent with a flow rate of 1 mL/min at 25 °C. The samples were identified by comparing the retention times with those of carbohydrate standards.

Surface activity measurement. Culture samples were centrifuged at 8000*g* for 20 min for cell removal and the supernatant was submitted to surface activity measurements. Surface and interfacial tension were determined with a Krüss Processor Tensiometer (model K12 T Krüss, Germany) using the plate method. Interfacial tension was performed against hexadecane. CMD^{-1} and CMD^{-2} were done by measuring the surface tension of 10-times and 100-times diluted broth in distilled water. The CMC was determined by measurements of surface tension of serial diluted broth samples as previous reported (Sheppard and Mulligan, 1987); the dilution factor obtained (CMC^{-1}) was related to the crude surfactant concentration (3 g/L after 48 h). Maximal standard deviation admitted to surface activity measurements was 0.20.

Biosurfactant isolation. Surfactant was obtained from cell-free broth by adjusting the broth pH to 2.0 using 6 N HCl and keeping it at 4 °C overnight. The

precipitate thus obtained was pelleted at 8000*g* for 20 min, dried and weighted. For further purification, the crude surfactant was dissolved in distilled water at pH 7.0 and dried at 60 °C. The dry product was extracted with chloroform:methanol (65:15), filtered and the solvent evaporated. The product thus obtained was used for TLC, biochemical composition and emulsification analysis.

Chemical characterization of biosurfactant. Preliminary characterization of the biosurfactant was done by thin layer chromatography (TLC). The components of chloroform/methanol extract were separated on silica gel 60 plates (Merck) using as solvent system $CHCl_3$:CH_3OH:H_2O (65:15:1). The components were detected by spraying the plate with distilled water and heating at 110 °C for 5 min (Makkar and Cameotra, 1997a). Surfactin (Sigma) was used as standard.

Biochemical analysis. The protein content of surfactant was estimated using the Biuret method (Gornall et al., 1949) and the lipid content by the method of Bligh and Dyer (1959).

Amino acid analysis. The amino acid composition of the surfactant was determined on a Pickering amino acid analyzer (Pickering Laboratories, USA) after total hydrolysis of the sample in 6 N HCl at 105 °C for 24 h.

Stability studies. Stability studies were done using cell-free broth obtained after 48 h of cultivation. Broth samples were heated in a boiling water bath for different time intervals and cooled at room temperature. The pH stability was performed by adjusting the broth to different pH values. For studying the effect of salt addition on biosurfactant, different concentrations of NaCl were added to broth samples and mixed until complete dissolution. The surface tension and CMD values of each treatment were performed as described above.

Emulsification activity was performed accordingly to Cooper and Goldenberg (1987), 6 mL of hydrocarbon was added to 4 mL of aqueous solution of biosurfactant (1 mg/mL), in a screw cap tube, and vortexed at high speed for 2 min. The emulsion stability was determined after 24 h, and the emulsification index (E_{24}) was calculated by dividing the measured height of emulsion layer by the mixture's total height and multiplying by 100.

Statistical analysis. The data are presented in terms of arithmetic averages of at least three replicates and the error bars indicate the standard deviations. The analyses were carried out using Microcal Origin software, version 6.0 (OriginLab Corporation, MA, USA).

3. Results

Carbohydrates contents and consumption are showed in Table 2. Sucrose is the main sugar present on cassava waste, although fructose and glucose are also present at significant concentrations. The decrease on sucrose levels and consequently increases on glucose and fructose, observed after 12 h, is due to sucrose hydrolysis. The presence of maltose suggests that soluble starch degradation takes place resulting on glucose and some maltose molecules. Sucrose was exhausted after 48 h and total carbohydrates were not completely consumed after 72 h.

Biosurfactant production and growth characteristics of *B. subtilis* LB5a using cassava wastewater as substrate are illustrated in Fig. 1. Maximal surfactant concentration (3 g/L) was attained after 48 h of cultivation and, at this point, most of the sugars were utilized and sucrose was exhausted. The lowest surface tension (ST) of 26.6 mN/m was attained after 12 h whereas CMD values continue to decrease. The interfacial tension of cell-free broth (after 48 h cultivation) against hexadecane was 0.97 mN/m.

Studies on the effect of heat treatment demonstrated that no appreciable change in surfactant surface activity had occurred (Fig. 2). The surface tension and CMD values remained stable after exposure to high temperatures (100 °C) even after 2 h. When submitted to autoclave sterilization (121 °C/15 min) the surface activity was also maintained. The surfactant surface properties remained stable for extended periods (6 months) under low temperature (−18 °C).

Table 2
Sugars consumption by *B. subtilis* LB5a growing on cassava wastewater

Time (h)	Carbohydrates concentration (g/L)				
	Sucrose[a]	Glucose[a]	Fructose[a]	Maltose[a]	Total carbohydrates[b]
0	22.0 ± 0.02	6.82 ± 0.01	4.51 ± 0.02	0	35.25 ± 1.54
12	1.13 ± 0.04	8.81 ± 0.03	6.60 ± 0.05	0.35 ± 0.03	21.20 ± 0.83
24	0.70 ± 0.03	2.08 ± 0.01	0.52 ± 0.04	0.78 ± 0.04	11.49 ± 0.76
36	0.53 ± 0.02	0	0.21 ± 0.04	0	4.24 ± 0.31
48	0.26 ± 0.02	0.45 ± 0.03	0.21 ± 0.03	0	4.07 ± 0.47
60	0	0	0.25 ± 0.04	0.34 ± 0.03	3.29 ± 0.23
72	0	0.48 ± 0.06	0.23 ± 0.04	0	3.32 ± 0.21

[a] HPLC.
[b] Phenol–sulfuric method.

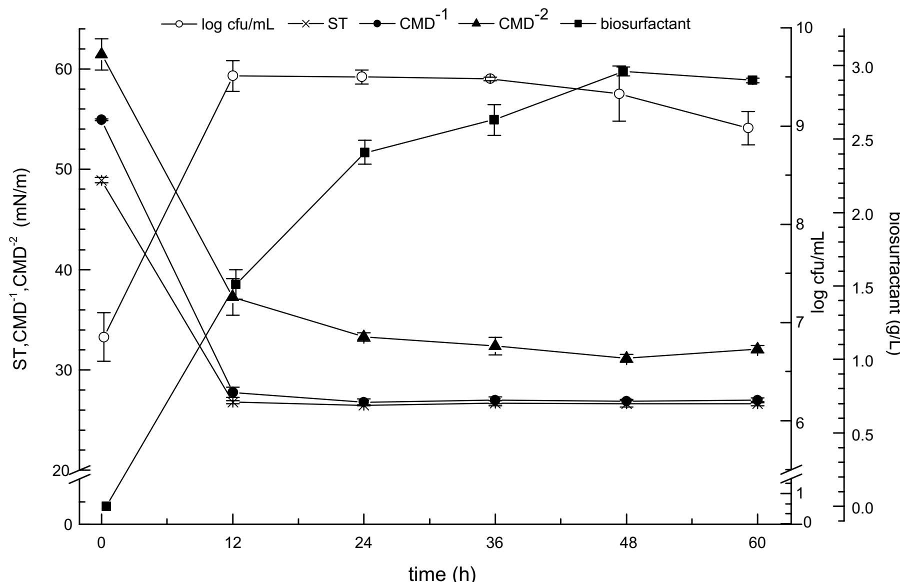

Fig. 1. Time-course of surfactant production by *B. subtilis* LB5a using cassava wastewater (ST: surface tension; CMD^{-1}, CMD^{-2}: critical micelle dilutions).

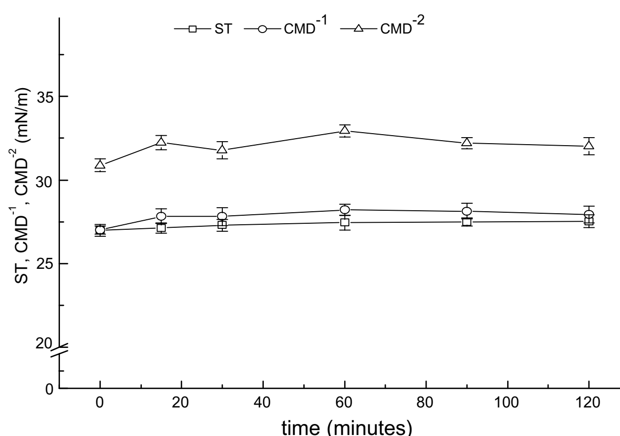

Fig. 2. Heat (100 °C) stability of the surfactant from *B. subtilis* LB5a broth (ST: surface tension; CMD^{-1}, CMD^{-2}: critical micelle dilutions).

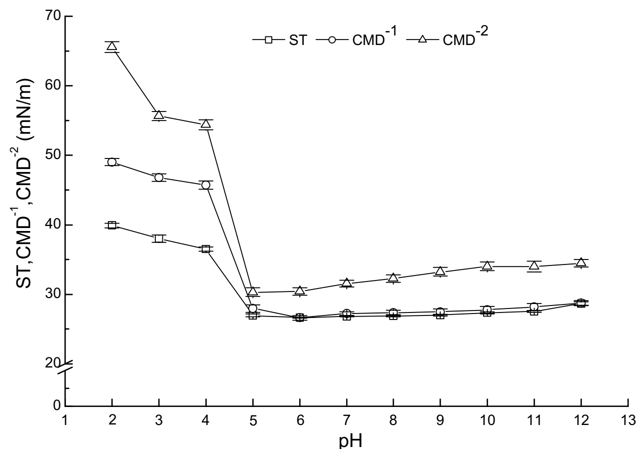

Fig. 3. Influence of pH on the surface activity of *B. subtilis* LB5a broth (ST: surface tension; CMD^{-1}, CMD^{-2}: critical micelle dilutions).

The effect of pH on biosurfactant activity is shown in Fig. 3. The surface activity was retained over a pH range of 5–11 with minimal deviation in surface tension and CMD^{-1}, whereas CMD^{-2} shown a slight and gradual increase on surface tension with increasing pH values. At lower end of pH scale (<5) surface tension was increased due to precipitation of surfactant.

Little changes were observed on surface tension and CMD values with the addition of up to 20% (w/v) sodium chloride (Fig. 4). Experimental results on the emulsification activity of biosurfactant obtained from *B. subtilis* on cassava medium are presented in Table 3. The product was capable of forming stable water-in-oil emulsions with all hydrocarbons tested.

Preliminary characterization of biosurfactant using TLC revealed a white dry spot with retention index of 0.56; the standard surfactin presented a retention index of 0.55. Biosurfactant obtained on cassava waste had a lipid content of 53.6% and a protein content of 38.5%. The analysis indicated the presence of four amino acid residues in the biosurfactant. The composition was determined to be glutamic acid, aspartic acid, valine and leucine in the ratios 1:1:1.2:3.4. The estimated critical micelle concentration (CMC) was 33 mg/L.

4. Discussion

The majority of known biosurfactants are synthesized from water-immiscible hydrocarbons, however, *B. subtilis* strains are able to produce surfactants from

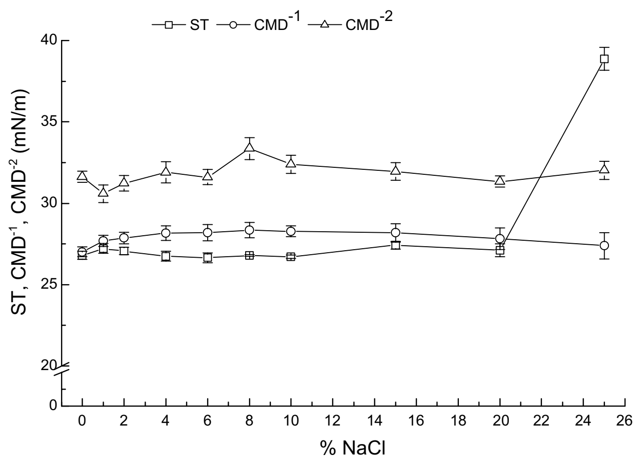

Fig. 4. Effect of salt concentration on the surface activity of *B. subtilis* LB5a broth (ST: surface tension; CMD^{-1}, CMD^{-2}: critical micelle dilutions).

Table 3
Emulsification activity (E_{24}) of biosurfactant against hydrocarbons

Hydrocarbon	E_{24} (%)
Hexane	66.6 ± 0.32
Toluene	72.7 ± 1.01
Heptane	66.6 ± 0.86
Decane	70.4 ± 0.54
Kerosene	70.4 ± 0.69
Tetradecane	70.9 ± 0.87
Hexadecane	69.0 ± 0.56
Soybean oil	74.0 ± 0.43
Coconut fat	70.9 ± 0.76
Crude oil	69.0 ± 0.41

water-soluble substrates, moreover, it was previously reported that the addition of a hydrocarbon to culture medium completely inhibited surfactin production by *B. subtilis* (Cooper et al., 1981). Water-soluble substrates are cheaper than hydrocarbons and are preferred because single-phase fermentation is simpler than biphasic fermentation (Makkar and Cameotra, 1997b).

Accordingly to Sandrin et al. (1990) glucose, fructose and sucrose were the best carbon substrates for the synthesis of surfactin and all these sugars are present on cassava wastewater. Molasses, a rich sucrose by-product was also suggested as an alternative medium for biosurfactant production by *B. subtilis* (Makkar and Cameotra, 1997b). Thompson et al. (2000) demonstrated that *B. subtilis* express α-amylase that permits the utilization of a starch-rich potato substrate as culture medium for biosurfactant production. The presence of maltose and also glucose at late fermentation times suggested an enzymatic starch hydrolysis, though soluble starch was present on cassava waste at low levels (0.4 g/L).

A direct relationship between biosurfactant production, cell growth and carbohydrates utilization was observed during the production of surfactant by *B. subtilis* C9 (Kim et al., 1997). The surfactant biosynthesis on cassava waste started at exponential growth phase and continued during the stationary phase, however, it can not be stated that biosurfactant production by *B. subtilis* LB5a is growth-associated, because 50% of biosurfactant was produced when cell growth reached stationary phase (Fig. 1). The cassava waste proved to be a suitable substrate for both bacterial growth and surfactant accumulation by the microorganism tested.

Surfactin, one of the most effective surfactant known so far, showed a surface tension of 25.0 mN/m, an interfacial tension <1.0 mN/m and a CMC of 25 mg/L (Cooper et al., 1981). The surfactant recovered from cassava waste had a surface tension of 26.6 mN/m, an interfacial tension of 0.97 mN/m and a CMC of 33 mg/L, characterizing the product as a powerful surface-active agent.

The composition of the medium, as reflected by nutrients balance, is of critical importance for determining product yield and surfactant properties (Sheppard and Mulligan, 1987) consequently; some characteristics and properties of biosurfactant obtained from cassava waste were studied. Stability studies of the product in culture broth indicated the surfactant to be thermostable and also pH stable from values over 5.0. Surfactant showed salt tolerance of up to 20%. These findings revealed that the product obtained could be very useful in situations where extreme conditions of temperature, salinity and alkaline pH are present, such as enhancing oil recovery and bioremediation of soil and marine environments.

The emulsification activity of biosurfactant revealed that it could be used as an emulsion-forming agent for hydrocarbons and oils, given stable emulsions. A surfactant obtained from *Rhodococcus* strain ST-5 had lower emulsifying index towards short-chain hydrocarbons than for long-chain hydrocarbons (Abu-Ruwaida et al., 1991). The emulsification activity of LB5a surfactant seemed not to be related to hydrocarbon chain length. The ability to form emulsions with vegetable oils and fats suggests potential application as cleaning and emulsifying agent in food industry.

The biosurfactant obtained from cassava wastewater showed high surface and interfacial tension reduction, small CMC values, and exhibited a high level of thermal stability is relatively stable to pH and demonstrates a high level of tolerance to ionic strength and good emulsification capacity suggesting potential commercial applications.

Preliminary chemical characterization of surfactant obtained suggested it to be a lipopeptide. The amino acids present were glutamic acid, aspartic acid, valine and leucine in the ratios 1:1:1.2:3.4. This composition is very similar to the amino acid composition of standard surfactin (Kakinuma et al., 1969). The complete structural characterization of the surfactant is been carried out. Cassava wastewater proved to be a suitable substrate for biosurfactant biosynthesis, providing not

only bacterial growth and product accumulation but also a surfactant that has interesting and useful properties.

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