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Characterization and properties of the biosurfactant produced by *Candida lipolytica* UCP 0988

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ABSTRACT

Background: Biotechnological processes are costly, especially for the production of biosurfactants. The successful production of a biosurfactant is dependent on the development of processes using low cost raw materials. Considering the importance of the characteristics of a biosurfactant to facilitate its industrial application, the properties of the biosurfactant produced by *Candida lipolytica* through previously optimized medium have been established.
Results: The yeast was grown for 72 h to determine the kinetics of growth and production. The surface tension of the cell-free broth was reduced from 55 to 25 mN/m. The yield of biosurfactant was 8.0 g/l with a CMC of 0.03%. The biosurfactant was characterized as an anionic lipopeptide composed of 50% protein, 20% lipids, and 8% of carbohydrates.
Conclusions: The isolated biosurfactant showed no toxicity against different vegetable seeds: *Brassica oleracea*, *Solanum gilo* and *Lactuca sativa* L. and the micro-crustacean *Artemia salina*. The properties of the biosurfactant produced suggest its potential application in industries that require the use of effective compounds at low cost.

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1. Introduction

Surfactants are amphipathic molecules that accumulate at interfaces, decrease interfacial tensions, and form aggregate structures such as micelles. Due to these properties, surfactants and emulsifiers are widely used for industrial, agricultural, food, cosmetic and pharmaceutical applications [1]. Surfactants can be derived from both petrochemical feedstock and renewable resources (plants and animal oils, micro-organisms). They were originally made from renewable resources like fats and oils, whereas today, the majorities are of petrochemical origin [2,3]. Some surfactants, known as biosurfactants, are biologically produced by yeasts or bacteria that have some effect on interfaces [4]. They are grouped as glycolipids, lipopeptides, fatty acids, polymeric and particulate compounds [5,6].

Biosurfactants have advantages over their chemicals counterparts because they are biodegradable, have low toxicity, are effective at extreme temperatures or pH values and show better environmental compatibility [7,8]. Nevertheless, from an economic standpoint, biosurfactants are not yet competitive with the synthetics. Biosurfactants can only replace synthetic surfactants if the cost of the raw material and the process is minimal [9,10].

In despite of the advantages, the economics of biosurfactant production have not received attention, but economic strategies must be devised if they are to compete with chemical surfactants [11,12]. They can be produced from various substrates, mainly renewable resources such as vegetable oils, distillery and dairy wastes, which are economical but have not been reported in detail [13]. The choice of low-cost substrates is important to the overall economics of the process because they account for 50% of the final product cost.

Key factors governing the success of biosurfactant production are the development of an economical process that uses low cost materials and gives high product yield and selected biosurfactants for specific applications. The aim of this work was to investigate the microbial transformation of a Brazilian industrial fatty waste from vegetable oil refineries by *Candida lipolytica* for biosurfactant production. The kinetics of biosurfactant production, its characterization, properties and toxicity were evaluated for the application of this biomolecule in environmental processes.

2. Materials and methods

2.1. Microorganism

The microorganism *C. lipolytica* UCP 0988 was kindly supplied from the Culture Collection of Nucleous of Research in Environmental

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Sciences, Catholic University of Pernambuco, Recife-PE, Brazil. The microorganism was maintained at the anamorph state at 5°C on Yeast Mold Agar (YMA) slants containing (w/v): 0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1% glucose and 2% agar. Transfers were made to fresh agar slants each month to maintain viability.

2.2. Cultural conditions and biosurfactant production

The production medium used for the experiments consisted of the following: 0.1% NH_4NO_3, 0.02% KH_2PO_4 and 0.02% $MgSO_4 \times 7H_2O$. The medium was supplemented with 6% soybean oil refinery residue and 1% glutamic acid. The refinery residue was obtained from ASA Indústria e Comércio LTDA (Recife-PE, Brazil). The composition of the refinery residue and the biosurfactant was produced as previously described by Rufino et al. [14]. After selection of the best medium composition and conditions of cultivation, kinetics of microorganism growth and biosurfactant production were monitored for 72 h. At regular intervals, different process parameters such as growth, pH, surface tension and biosurfactant concentration were determined.

2.3. Biomass determination

For biomass determination, 5 ml samples were mixed in preweighed tubes with chilled distilled water and centrifuged at 5000 rpm for 20 min. After two washing cycles the cell pellet was dried in an oven at 90°C for 24 h. All the assays were carried out in triplicate and did not vary more than 5%.

2.4. Isolation of biosurfactant

The 72 h culture was filtered through a Whatman no. 1 filter paper and centrifuged at 5000 × *g* for 20 min. The cell-free broth was concentrated (500 ml) by freeze drying and extracted two times with chloroform (1:1, by vol.) in a separatory funnel at 28°C [15].

2.5. Surface tension and Critical Micelle Concentration (CMC)

The surface tension was measured by the ring method using a DuNouy Tensiometer model Sigma 700 (KSV Instruments LTD, Finland) at room temperature. The concentration at which micelles began to form was represented as the Critical Micelle Concentration (CMC). At the CMC, sudden changes in surface tension, electrical conductivity and detergency were observed. The CMC was automatically determined by measuring the surface tensions of the purified biosurfactant in distilled water up to a constant value of surface tension.

2.6. Phytotoxicity assay

The phytotoxicity of the biosurfactant was evaluated in a static test by seed germination and root elongation of cabbage (*Brassica oleracea*, *Solanum gilo*, *Lactuca sativa* L.) according to Tiquia et al. [16]. Solutions of the isolated biosurfactant were prepared with distilled water in concentrations of 0.15, 0.3 and 0.6 g/l. The toxicity was determined in sterilized Petri dishes (1 cm × 10 cm) containing a Whatman no. 1 filter paper. The seeds were pre-treated with sodium hypochlorite and 10 seeds were inoculated in each Petri dish which was inoculated with 5 ml of the test solution at 27°C. After five days of incubation in the dark, the seed germination, root elongation (≥5 mm) and germination index (GI, a factor of relative seed germination and relative root elongation) were determined as follows:

$$\text{Relative seed germination (\%)} = (\text{number of seeds germinated in the extract} / \text{number of seeds germinated in the control}) \times 100$$

$$\text{Relative root length (\%)} = (\text{mean root length in the extract} / \text{mean root length in the control}) \times 100$$

$$\text{Germination index} = [(\%\text{of seed germination}) \times (\%\text{of root growth})]/100\%.$$

Controls were prepared with distilled water to replace the biosurfactant solutions. The mean and standard deviation of triplicate samples from each concentration were calculated.

2.7. Artemia assay

The toxicity assay was performed with the isolated biosurfactant using brine shrimp (the microcrustacean *Artemia salina*) as the toxicity indicator. Brine shrimp eggs were obtained in a local store. Larvae were used within 1 day of hatching. Following dilutions of a biosurfactant solution at the 1/2 CMC, CMC and 2 × CMC (0.15, 0.3 and 0.6 g/l) with saline water (33 mg/l), the assays were conducted in penicillin tubes of 10 ml capacity containing 10 brine shrimp larvae in 5 ml of saline water per tube. The cell-free broth containing the biosurfactant was also tested. The brine shrimp larvae in each tube were tested using 5 ml per concentration level of biosurfactant solution. They were observed for 24 h to calculate mortality [17]. The toxicity threshold concentration, expressed as biosurfactant concentration per 100 ml of saline water, was defined as the lowest concentration that killed all brine shrimp within 24 h. Each test was run in triplicate, and saline water was used as the control.

2.8. Biosurfactant characterization

Protein concentration in the isolated biosurfactant was estimated by using the total protein test kit from Labtest Diagnóstica S.A., Brazil. The total carbohydrate content was estimated by the phenol-sulphuric acid method [18]. The lipid content was determined according to Manocha et al. [19]: 0.5 g of the isolated material was extracted with chloroform: methanol in different proportions (1:1 and 1:2, v/v). The organic extracts were then evaporated under vacuum and the lipid content determined by gravimetric estimation.

2.9. Determination of biosurfactant fatty acid composition

The fatty acid composition of crude biosurfactant extract was analysed by GC-FID (CP-3380, Varian Inc., California, USA) of fatty acids methyl ethers, as described by Durham and Kloos [20]. The analyses were carried out in a gas chromatograph equipped with the capillary column HR-SS-10. The carrier gas was helium at 24 ml/min. The injector and detector (FID) temperature was 250°C, oven temperature at 130°C, starting and increasing to 170°C at 1°C/min, to 180°C at 3°C/min, kept isothermically for 10 min. The chromatographic peaks were identified by comparing with the chromatogram of the standard fatty acid methyl mixture (Sigma Chemical Co., St. Louis, MO, USA).

2.10. Determination of biosurfactant ionic character

The ionic charge of the biosurfactant was determined using the agar double diffusion technique [21]. Two regularly spaced rows of wells were made in an agar of low hardness (1% agar). Wells of one row were filled with the biosurfactant solution and wells of the other were filled a pure compound of known ionic charge. The anionic substance chosen was sodium dodecyl sulphate (SDS) 20 mM and the cationic one was barium chloride, 50 mM. The appearance of precipitation lines between the wells, indicative of the ionic character of the biosurfactant, was monitored over a 48 h period at ambient temperature.

3. Results and discussion

3.1. Fermentation kinetics of the biosurfactant produced by *Candida lipolytica*

The prospect for producing biosurfactants by yeasts largely depends on identifying an inexpensive and abundant feedstock. The objective in the commercial production of surfactants can be described as "optimum quality and quantity at minimum cost" [22].

A potential source of substrates and the least expensive is waste streams. In addition, waste treatment costs can be offset by the production of valuable co-products. Such carbon sources can often be obtained at little or no cost [23]. The economics of biosurfactant production using inexpensive raw materials can make biosurfactant processes competitive with the chemical ones. In most instances, the carbon source accounts for 50% of the final production cost of a bioprocess [22].

The *C. lipolytica* was cultivated in optimized medium containing 6% soybean oil refinery residue and 1% glutamic acid. As shown in Fig. 1, growth started without a lag time and stopped after about 48 h, when biomass reached 11 g/l. During the first 12 h, the pH practically did not vary, but after that point it started to rise, reaching 7.0 after 72 h. The surface tension of the culture broth (50 mN/m) dropped rapidly after inoculation, reaching its lowest value (25 mN/m) during the exponential phase after about 16 h, and remaining stable after that.

Maximum biomass concentration was achieved after 50 h, when the yeast entered the stationary growth phase. The surface tension dropped rapidly from around 50 mN/m to 25 mN/m in the early stages of the exponential growth phase, indicating excellent surface-active properties, while pH remained practically unchanged during the cultivation period. The results show that the highest biosurfactant production occurred during the exponential phase and stationary growth.

Most biosurfactants are excreted into the culture medium either during the exponential phase or at the stationary phase [23]. Cirigliano and Carman [15] showed that the emulsifier production by *C. lipolytica* IA 105530 was detected when microorganism growth rate decreased but Amaral et al. [24] described a growth-associated bioemulsifier production by *Yarrowia lipolytica* IMUFRJ 50682 using a different carbon source.

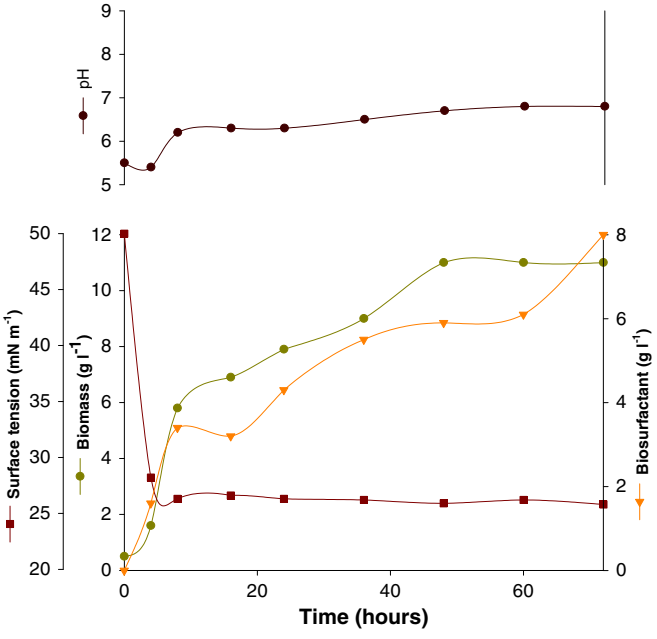

Fig. 1. Growth, pH and surface active profile of *C. lipolytica* grown in mineral medium with 6% industrial residue as substrate and 1% glutamic acid.

Torulopsis bombicola produced most of the surfactant in the late exponential phase of growth. Therefore, Cooper and Paddock [25] proposed to grow the yeast on a single carbon source and then add a second type of substrate after the exponential growth phase, causing a burst of glycolipids production. The maximum yield was 70 g/l of the weight of the substrate used [2].

Studies have shown that the medium acidity is related with a minor surfactant production [26]. Considering that the pH was not controlled in this study and that it was only slightly altered during cultivation, it is possible that this behaviour contributes for the obtention of high yields in biosurfactants, as observed by Rufino et al. [22] when *C. lipolytica* UCP 0988 was cultivated in an industrial residue.

According to the literature, the biosurfactants produced by bacteria strains are more effective in reducing the surface tension. *Pseudomonas aeruginosa*, as an example, is known as one of the most studied strain for the production of biosurfactants. Most of the biosurfactants produced by this bacterium have shown to reduce the surface tension to values around 27,28 mN/m [27,28,29]. Although the yeasts biosurfactants described in the last few decades have shown capacity to reduce the surface tension to values around 35 mN/m [30], researches revealed similar values to those obtained by bacteria, as the result obtained in this work for the biosurfactant from *C. lipolytica*.

Regarding the use of food grade vegetal oils, waste frying vegetal oils or residues of vegetal oil refinery, isolated or combined with a soluble substrate, promising results have been obtained in the last decade for *Candida* species in our laboratories [5,22,31]. It is important to consider, however, that the substrates act differently for a specific microorganism, as pointed out by Haba et al. [32], who observed that used olive oil was not a suitable substrate for cell growth, although the surface tension of the supernatant fluid decreased with *Candida* sp. 39A2 (35 mN/m), *Candida albicans* (39 mN/m), *Candida rugosa* IFO0750 (39 mN/m) and *Candida tropicalis* CECT 1357 (35 mN/m). In this case, *C. lipolytica* (43 mN/m) and *Candida torulopsis* (45 mN/m) were poor producers. Sunflower oil, on the other hand, supported good cellular growth in most cases and surface tension decreased as follows: *Candida* sp. 39A2 (35 mN/m), *C. rugosa* IFO0750 (39 mN/m), *C. lipolytica* and *C. torulopsis* (40 mN/m).

On the other hand, the minor surface tension obtained in the cell-free broth of *Candida antarctica* cultivated in a medium with 10% soapstock was 35.7 mN/m [26]. According to Bednarski et al. [26], the difficulty in interpreting the changes in the surface tension of metabolic broth is related to many factors including varied composition of fatty residues, and further, with their varied bioconversion by yeast.

3.2. Biosurfactant yield

Candida strains have been shown to produce biosurfactants with different yields, which are dependent of the medium and culture conditions. Crude biosurfactant of 8 g/l was extracted from the 72 h culture of *C. lipolytica* UCP 0988 showing a growth-associate production.

A biosurfactant yield of 4.5 g/l was extracted from the 144 h culture of *Candida sphaerica* [33]. On the other hand, the yield of another biosurfactant produced by *C. sphaerica* was 9 g/l after 144 h of experiment [34], which is in accordance with the values previously reported in the literature [35]. Sarubbo et al. [5] reported a yield of 8 g/l for a biosurfactant produced by *C. lipolytica* using canola oil and glucose as substrates.

3.3. Surface tension and Critical Micelle Concentration (CMC) of the biosurfactant

The surface-active properties of biosurfactant mainly depend on its ability to lower surface and interfacial tensions, CMC value and formation of stable emulsion. The ability to reduce the surface tension depends on the specific concentration of surface-active compound, *i.e.*, the CMC which is defined as the minimum concentration of

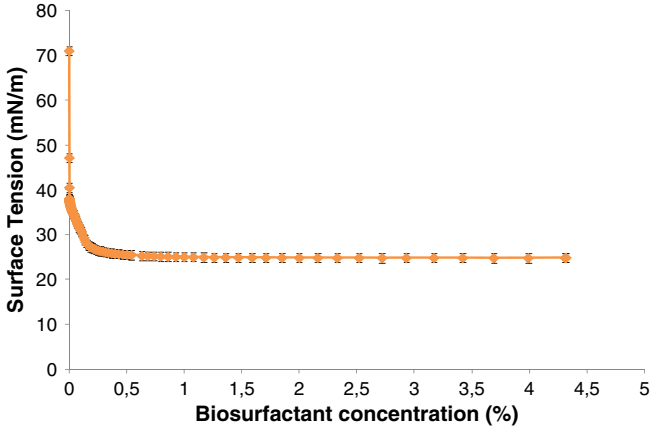

Fig. 2. Surface tension versus concentration of the purified biosurfactant.

biosurfactant required to give maximum surface tension reduction of water and initiate micelle formation. Efficient surfactants have very low CMC values, *i.e.*, less surfactant is required to decrease surface tension [36,37]. Surface tensions versus the isolated biosurfactant concentrations were plotted in Fig. 2. The surface tension of water decreased gradually with increasing biosurfactant concentration from 70 to 25 mN/m, with a biosurfactant concentration of 0.03%, and then remained constant. The CMC of the crude biosurfactant is within the range of CMC values reported for different types of biosurfactants produced by other *Candida* species [9,14,36,38].

Members of the genus *Candida* have been recently shown to produce highly effective biosurfactants able to reduce the surface tensions of water to low values [5,9,14,35]. Such values are lower than those of synthetic surfactants in the partially purified and even in cell free broth preparations containing biosurfactants.

The biosurfactant produced showed also a smaller CMC value than those of other biosurfactants from yeasts described in the literature, as values of 2.5% found for biosurfactants from *C. lipolytica* and *Candida glabrata* [38], and of 0.6% for the biosurfactant from *C. antarctica* [39].

The biosurfactant from *C. lipolytica* showed a lower minimum surface tension than that of another biosurfactant from *C. lipolytica* (32 mN/m) [22], and that of the biosurfactants from *C. glabrata* (31 mN/m) [5], from *C. antarctica* (35 mN/m) [39] and from *Y. lipolytica* (50 mN/m) [24].

3.4. Biosurfactant toxicity

The biosurfactant from *C. lipolytica* was first tested for its toxicity in a short term bioassay using brine shrimp. These results indicate the low toxicity of the biosurfactant from *C. lipolytica* UCP0992.

The germination index (GI), which combines measures of relative seed germination and relative root elongation, has been used to evaluate the toxicity of the biosurfactant on cabbage (*B. oleracea*). Considering that a GI value of 80% has been used as an indicator of the disappearance of phytotoxicity [16], the results obtained indicated that the solutions tested did not show inhibitory effects on the seed germination and root elongation of cabbage, since GI of 136% was found for biosurfactant solutions of 60 mg/l. It was also possible to visualize the leaves and the elongation of secondary roots for all the conditions tested. Similar results were observed by Luna et al. [40] for the biosurfactant produced by *C. sphaerica*.

3.5. Preliminary characterization of biosurfactant

Preliminary chemical characterization of biosurfactant revealed that the examined agent was a lipoprotein material which consisted of protein (50%), lipid (20%) and carbohydrate (8%). Differently, the emulsifier Liposan produced by *C. lipolytica* grown in hexadecane as substrate is composed of 83% carbohydrate and 17% of protein [41] and glycolipid produced by *C. sphaerica* consisted of 70% lipids and 15% carbohydrates [40].

Other biopolymers are produced in our laboratory from *Candida* and the surfactants produced by this genus can differ widely from one species to another. It has been reported to produce sophorose lipids [42,43], lipid–carbohydrate complexes [44], protein–carbohydrate complexes [41], long-chain fatty acids [45] and protein–carbohydrate–lipid complexes [5,22,31,36,46,47] when grown either on hydrophobic or water-miscible substrates.

In this study the fatty acid composition of the isolated biosurfactant analysed by Gas Chromatography revealed the presence of C12:0 (75.34%) as the major component and, C8:0 (7.96%), C18:1 (6.36%), C16:1 (4.23%), C14:0 (3.85%) and C16:0 (2.25%) in minor quantities. The results obtained for the biosurfactant from *C. glabrata* from GC analysis differ from the one obtained here, indicating the presence of the following fatty acids: C18:1 (55%), C16:0 (15%), C12:0 (21%) and C18:0 (9%) [44].

Agar double diffusion tests revealed the appearance of precipitation lines between the biosurfactant produced by *C. lipolytica* and the cationic compound selected (barium chloride), while no lines had formed between the biosurfactant and the anionic compound (SDS). Under the experimental conditions of this work, this very simple test demonstrated the anionic character of the biosurfactant produced. Other biosurfactants produced by *Candida* species also display an anionic character when submitted to the same test [40].

4. Concluding remarks

The key factor governing the success of biosurfactant production is the development of an economical process that uses low-cost materials and gives high productivity. To enhance the efficiency of biosurfactant production by microorganisms, inexpensive medium components such as food industry by-products or waste should be explored, since they represent about 50% of the total production costs. In the present study, a low cost fermentative medium based on industrial residue as substrate was successfully evaluated for lipopeptide production by the yeast *C. lipolytica*. The combination of the residue with glutamic acid resulted in high biosurfactant production by yeast. The excellent surface tension reducing property, CMC and the absence of toxicity suggest the possibility of the use of this new biosurfactant in a wide variety of industrial, environmental and biotechnology applications.

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