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Biosurfactant Production from *Candida Guilliermondii* and Evaluation of its Toxicity

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The rapid development of biotechnology and the increase in environmental awareness among producers and consumers have placed biological products in a preferential position on the market. The physiochemical properties of biosurfactants make these natural compounds an attractive option with potential use in a variety of industrial and biotechnological applications as additives in foods, cosmetics and detergents. Thus, the aim of the present study was to select the better biosurfactant-producing microorganism between two species of Candida (*C. guilliermondii* UCP0992 and *C. lipolytica* UCP0998) grown in media with different sources of carbon (glucose, sugarcane molasses and waste frying oil) and nitrogen (yeast extract, urea and corn steep liquor). After the selection of the microorganism and production medium and biosurfactant production were analyzed. The properties of the biopolymer were then studied in terms of toxicity, using the brine shrimp *Artemia salina*, vegetable seeds and onion (*Allium* cepa) as bioindicators. Gas chromatography was performed for the structural characterization of the biomolecule and the determination of its physicochemical composition. *Candida guilliermodii* grown in a medium with 5.0% molasses, 5.0% corn steep liquor and 5.0% waste frying oil was selected for the production of biosurfactant, with a reduction in surface tension to 28.6 mN/m, a yield of 21 g/l and a critical micelle concentration of 0.7 g/l. The isolated biosurfactant was not toxic to *Artemia salina,* or the vegetable seeds tested. The biosurfactant had a glycolipid nature. Based on the present results, the novel biosurfactant produced herein has potential application in industrys.

1.Introducion

In the era of globalization, many classic industries in the quest for innovation have increasing turned to biotechnology, which has enabled diverse research opportunities without exerting a negative effect on productivity (Campos et al., 2015). In this context, biologically produced surfactants (biosurfactants) are an attractive option, offering several advantages over their synthetic counterparts, such as the ability to be produced from industrial waste products, biodegradability, tolerance to a wide range of environmental conditions and comparatively lower toxicity (Geetha; Banat; Joshi, 2018). Considering the growing interest in alternative products that minimize environmental impacts (Brumano, 2017). Yeasts of the genus *Candida* stand out among species of biosurfactat-producing microorganisms due to their usefulness products (Bourdichon et al., 2012). Studies have investigated the production of biosurfactat from C. lipolytica using regional oily substrates or glucose (Santos et al., 2013). *C. bombicola* and *C. lipolytica* are among the most commonly studied yeasts for the production of biosurfactants (Santos et al., 2017). Thus, the aim of the present study was to produce a non-toxic biosurfactant with for application in industrys

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2. Materials and Methods

2.1. Microorganisms

Two species of *Candida* (*C. guilliermondii* UCP 0992 and *C. lipolytica* UCP 0988) were tested as biosurfactant producers. Both strains were acquired from the culture bank of the Environmental Science Research Center of the Catholic University of Pernambuco.

2.2. Maintenance medium

The yeasts were maintained at 5°C in yeast mold agar (YMA) with the following composition (p/v): yeast extract (0.3%), malt extract (0.3%), tryptone (0.5%), D-glucose (1%) and agar (5%), dissolved in distilled water (100 ml). Subcultures were performed monthly to maintain cell viability.

2.3. Growth medium for inoculum

Yeast mold broth (YMB) was used for growth of the inoculum, which has the same composition as YMA with the exception of agar (p/v): yeast extract (0.3%), malt extract (0.3%), tryptone (0.5%) and D-glucose (1%). All components were solubilized in distilled water and the medium was sterilized in an autoclave at 121°C for 20 minutes.

2.4. Preparation of inoculum

Inocula were transferred to a tube containing YMA medium to obtain a young culture. Next, the sample was transferred to flasks containing 50 ml of YMB medium, followed by incubation with constant stirring at 200 rpm and 28°C for 24 horas. After this period, cell counts were performed in a Neubauer chamber until obtaining the desired final concentration of cells (10^8 cells/ml).

2.5. Biosurfactant production media

Different media were tested for the production of biosurfactants: A) 5.0% glucose, 0.1% yeast extract, 0.1% urea and 5.0% waste frying oil; B) 5.0% molasses, 0.1% yeast extract, 0.1% urea and 5.0% waste frying oil; C) 5.0% molasses, 5.0% corn steep liquor and 5.0% waste frying oil; and D) 2.5% molasses, 2.0% corn steep liquor and 2.5% waste frying oil.

2.6. Production of biosurfactant

Fermentations for the production of biosurfactant were performed in 1000-ml Erlenmeyer flasks containing containing 500 ml of production medium and incubated with the suspension of 106 cells/ml. The inocula were added and the media were kept under orbital stirring at 200 rpm for 144 hours at a temperature of 28°C. After the incubation period, the media were submitted to centrifugation with stirring at 4500 rpm for 20 minutes for the obtainment of the cell-free broth. Aliquots were withdrawn after fermentation for the determination of surface tension, biomass, pH and yield of biosurfactant.

2.7. Determination of surface tension

Surface tension of the biosurfactant was measured in the cell-free broth with the aid of an automatic tensiometer (KSV Sigma 70, Finland), using the du Noüy ring method.

2.8. Determination of emulsification activity

For the determination of emulsification activity, the samples were centrifuged at 4500 rpm for 15 minutes and analyzed using the method proposed by Cooper and Goldenberg (1987).

2.9. Isolation of biosurfactant

Two different extraction methods were tested for the isolation of the biosurfactant produced by *C. guilliermondii*. The method developed in the laboratory involved ethyl acetate and the cell-free broth with the non-centrifuged medium at a proportion of 1:4 (repeating twice). Next, the organic phase was submitted to centrifugation (4500 rpm for 20 minutes) followed by filtration. The filtered sample was transferred to the separation funnel and a saturated solution of sodium chloride (NaCl) was added to separate the remaining aqueous phase. The organic phase was transferred to a beaker and set to dry on a hot plate at a temperature of 60°C. Next, sodium hydroxide (NaOH) and acetone (C_3H_6O) were added until the formation of granules, followed by paper filtration and drying at a temperature of 50 °C.

For methanol extraction after 144 h of *C. guilliermondii* culturing, the broth was centrifuged at 2000 rpm for 20 min for the removal of the cells and submitted to the extraction process. The pH was adjusted to 2 with a solution of HCl 6.0 M and precipitated with two volumes of methanol. After resting for 24 horas at -15ºC, the

samples were centrifuged at 4000 g and 28ºC for 30 min, washed twice with cooled methanol, dried at 37ºC for 48 horas and kept in a desiccator until reaching a constant weight. The yield of the isolated product was expressed as g/l.

2.10. Determination of critical micelle concentration (CMC)

To determine the CMC, 0.1 g of isolated biosurfactant was diluted to an initial concentration of 5 g/l, followed by successive dilutions with distilled water. The surface tension of the respective dilutions was quantified using the du Noüy ring method.

2.11. Phytotoxicity test

The phytotoxicity of the biosurfactant was evaluated in a static assay involving seed germination and root growth of three vegetable plants: cabbage (*Brassica oleracea*), tomato (*Solanum lycopersicum*) and maroon cucumber (*Cucumis anguria*), based on Tiquia et al. (1996). Test solutions were prepared in distilled water with biosurfactant concentrations of 1/2 x CMC, 1 x CMC and 2 x CMC (0.35, 0.7 and 1.4 g/l, respectively). After five days of incubation in the dark, seed germination, root growth (≥ 5 mm) and the germination index were calculated using the following formulas:

(1) Relative seed germination (%) = (nº of seeds germinated in extract / nº of seeds germinated in control) x 100

(2) Relative root length (%) = (mean root length in extract / mean root length in control) x 100

(3) Germination index = [(% of seed germination) x (% of root growth)] / 100%

2.12. Toxicity assay with *Artemia salina*

The toxicity assay was performed with the isolated biosurfactant using brine shrimp (*Artemia salina*) as the bioindicator. Brine shrimp eggs were obtained from the local market. The larvae were used within one day after hatching. After dilutions of the biosurfactant solution at 1/2 x CMC, 1 x CMC and 2 x CMC (0.35, 0.7 and 1.4 g/l, respectively) in seawater, the assays were performed in a 50-ml beaker with 10 larvae in 20 ml of seawater + 20 ml of the biosurfactant at the different concentrations. The mortality rate was calculated after 24 hours. The toxicity threshold (expressed as the concentration of biosurfactant per 100 ml of seawater) was defined as the lowest concentration that killed all brine shrimp within 24 h. Each test was performed in duplicate using seawater as the control.

2.13. Toxicity test with onion (*Allium cepa L.*)

Toxicity of the isolated biosurfactant was also evaluated using onion (*Allium cepa* L.) as the indicator. The biosurfactant was used at concentrations of 1/2 x CMC, 1 x CMC and 2 x CMC (0.35, 0.7 and 1.4 g/l, respectively). The cell-free broth was also tested. The root growth inhibition test was conducted using the method described by Jardim (2004), with some modifications. These tests were performed in triplicate.

2.14. Gas chromatography (GC)

The sample of fatty acids (hydrophobic fraction) of the biosurfactant was analyzed in a gas chromatograph (Hewlett Packard model HP 5890 Série II) with an injector temperature of 220 ºC. Chromatographic separation was performed in a DB-5 column (30 m x 0.32 mm x 0.5 µm) with a flame ionization detector (FID) at 290 ºC, using nitrogen as the carrier gas. The initial temperature was programmed at 60ºC with a rate of 7°C/min until a final temperature of 200ºC. One µL was injected using hexane as the solvent.

3. Results and Discussion

3.1. Selection of culture medium and biosurfactant-producing microorganism

In the present study involving two strains of *Candida* grown in different production media, the biosurfactants produced by *C. lipolytica* led to surface tensions ranging from 33 to 52 mN/m. The biosurfactant produced by *C. guilliermondii* grown in 5% molasses, 5% corn steep liquor and 5% waste frying oil reduced the surface tension of water from 72 to 28.6 mN/m. This condition was therefore selected and the biosurfactant produced by *C. guilliermondii* demonstrated excellent surface tension-reducing capacity. Luna et al. (2016) and Sarubbo et al. (2015) obtained similar surface tension results (27 and 32 mN/m).

According to Luna et al. (2013), surfactants capable of lowering the surface tension of water from 72 to 35 mN/m are considered good tensioactive agents.
Comparing the surface tension of the medium achieved with the biosurfactant produced by *C. guilliermondii* to values reported by Almeida et al. (2017) using *C. tropicalis* grown in a medium containing 2.5% sugarcane molasses, 2.5% waste frying oil and 2.5% corn steep liquor, the results are quite similar: 34.12 ± 0.07 mN.m-1 in a 2l bioreactor and 35.60 ± 0.05 $mN.m^{-1}$ in a 50 l bioreactor. Daylin et al. (2017) report even better results for a biosurfactant produced by *C. tropicalis*, with a reduction in the surface tension of water from 72 to 28.5 mN/m. Moreover, the biosurfactant from *C. tropicalis* exhibited a greater compacity to reduce surface tension in comparison to biosurfactants from *C. glabrata* (31 mN/m) and *C. lipolytica* (32-33 mN/m).

3.2. Extraction methods and biosurfactant yield

Two biosurfactant extraction methods were tested. The best yield (21 g/l) was achieved using ethyl acetate, followed by extraction using acidic precipitation (4.3 g/l). What in Santos et al. (2013), obtained the result of 8 g/l for extraction with ethyl acetate. With ethyl acetate as the extraction solvent, it was possible to obtain the biosurfactant in its oily form with a lighter color compared to the method with methanol, with which the biosurfactant was oily and granular, but darker. Regarding viability, the method developed in the laboratory with ethyl acetate was the better choice, as it requires a smaller volume of solvent without the need for initial steps of centrifugation and filtration, obtaining an oily, limpid extract, which is considered more adequate for subsequent applications in food formulations.

3.3. Determination of critical micelle concentration (CMC)

A surfactant has the capacity to reduce surface tension (air/water) and this reduction is proportional to the concentration of the biosurfactant in the solution until reaching the CMC (Daltin, 2012). As shown in Figure 3, the CMC of the biosurfactant produced by *B. cereus* was approximately 33 mN/m, which falls within the range considered promising for the production of a biosurfactant (28 to 35 mN/m) (Santos et al., 2016).
The CMC is the minimum concentration of a biosurfactant necessary for the maximum reduction in the surface tension of water and the onset of the formation of micelles. This concentration is used as a measure of the efficiency of a biosurfactant. A good biosurfactant should exhibit high efficiency and efficacy at concentrations less than 1 g/l. In the present study, the surface tension of water was gradually reduced from 72 to 30 mN/m with the increase in the concentration of the isolated biosurfactant. Beyond 0.7 g/l, no additional reduction in surface tension was found with the further increase in the concentration of the biosurfactant, indicating that the CMC had been reached.
Based on the results, the biosurfactant produced by *C. guilliermondii* grown in a medium with 5% molasses, 5% corn steep liquor and 5% waste frying oil was chosen for the subsequent isolation study, characterization and use in the mayonnaise formulations.

3.4. Phytotoxicity test

Toxicity is defined as the capacity of a substance to have a harmful effect on a living organism. This depends on the concentration and properties of the chemical product to which the organism is exposed as well as the exposure time (Santos et al., 2016). The toxicity of the biosurfactant from C. guilliermondii to three vegetable species is displayed in Table 2.

Table 2: Phytotoxicity of isolated biosurfactant from C. guilliermondii grown in medium formulated with 5% molasses, 5% waste frying oil and 5% corn steep liquor to the seeds of three vegetable species

Vegetable seeds	**Germination index**		
	Isolated biosurfactant at 0.35%	Isolated biosurfactant at 0.7%	Isolated biosurfactant at 1.4%
Cabbage (*Brassica oleracea*)	100%	64%	49%
Maroon cucumber (*Cucumis anguria*)	72%	100%	97%
***Tomato (Solanum lycopersicum*)**	43%	57%	89%

The toxicity of the biosurfactant from *C. guilliermondii* was tested in a short bioassay. Bioassays involving plants play an important role in predicting the effect of chemical products in an ecosystem.
The germination index, which combines measures of relative seed germination and relative root growth, was used to evaluate the toxicity of the biosurfactant to the seeds of *Brassica oleracea*, *Cucumis anguria* and *Solanum lycopersicum*. The results revealed that the solutions tested had no inhibitory effect on seed germination or root growth, indicating low toxicity of the biosurfactant. A germination index of 80% was used as an indicator of the absence of phytotoxicity (Meylheuc et al., 2001). Moreover, leaf growth and the growth of secondary roots occurred under all conditions tested.

3.5. Toxicity assay with Artemia salina

Brine shrimp, Artemia salina, is commonly used in exotoxicology due to its simplicity in terms of laboratory handling and its short life cycle (Meyer et al., 1982). After exposure to the biosurfactant at ½ x CMC, 1 x CMC and 2 x CMC for 24 h, the *Artemia salina* larval survival rate was 100%. This finding demonstrates the low toxicity of the biosurfactant under the conditions tested. Luna et al. (2013) and Rufino et al. (2014) report similar results for other biosurfactants.

3.6. Structural analysis

The GC analysis of the fatty acid composition of the isolated biosurfactant revealed C16:0 as the main component (75.3%), followed by C17:0 (19.6%) and C18:1 (5%) (Figure 6). The biosurfactant has diverse fatty acids in different proportions, demonstrating potential application in industrys. Based on the results, the isolated biosurfactant has a glycolipid nature.

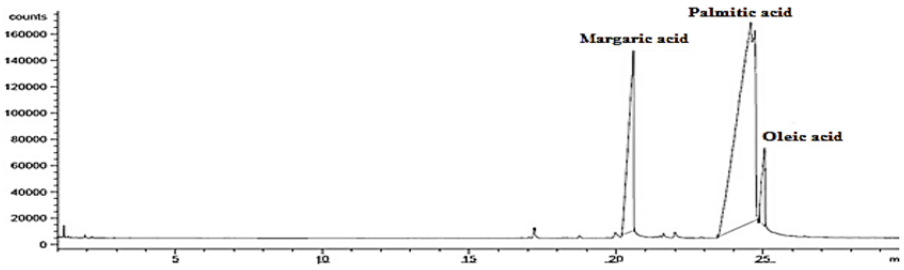

Figure 1: Chromatogram of fatty acid profile of biosurfactant produced by C. guilliermondii in medium supplemented with 5% molasses, 5% corn steep liquor and 5% waste frying oil.

4. Conclusion

The novel biosurfactant of a glycolipid nature produced by C. guilliermondii in a low-cost medium containing waste frying oil, molasses and corn steep liquor exhibited promising properties, such as the reduction of surface tension to 28.6 mN/m and low toxicity. Therefore, this biosurfactant has potential application, constituting a promising ingredient for use in the industry.

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