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Antimicrobial and anti-adhesive potential of a biosurfactant Rufisan produced by *Candida lipolytica* UCP 0988

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ABSTRACT

In the last years, researches developed with biosurfactants for application in the medical area have been revealing the promising biological activities of these biomolecules. In this work the antimicrobial and anti-adhesive properties of a biosurfactant Rufisan isolated from the yeast *Candida lipolytica* UCP 0988, growth in a medium supplemented with ground nut refinery residue was determined against several microorganisms. The biosurfactant was able to reduce the water surface tension from 70 to 25.3 mN/m and showed a critical micelle concentration (CMC) of 0.03%. The biosurfactant was isolated after 72 h of fermentation and was tested in concentrations varying from 0.75 to 12 mg/l. The highest antimicrobial activities were observed against *Streptococcus agalactiae*, *Streptococcus mutans, Streptococcus mutans* NS, *Streptococcus mutans* HG, *Streptococcus sanguis* 12, *Streptococcus oralis* J22 at a concentration superior to the biosurfactant critical micelle concentration. Moreover, the biosurfactant showed anti-adhesive activity against most of the microorganisms tested. As far as we know, this is the first compilation of data on antimicrobial and anti-adhesive activities of a biosurfactant obtained from a *Candida* strain against such a broad group of microorganisms. The results obtained in this work showed that the biosurfactant from *C. lipolytica* is a potential antimicrobial and/or anti-adhesive agent for several biomedical applications.

1. Introduction

Surfactants, amphiphilic molecules consisting of a polar head group and a hydrophobic tail, are the active ingredients found in soaps and detergents. Due to their ability to concentrate at the air–water interface, they are commonly used to separate oily materials from a given medium. Surfactants increase the aqueous solubility of hydrophilic molecules by reducing their surface/interfacial tension at air–water and water–oil interfaces [1,2]. As the interfacial tension is reduced and the aqueous surfactant concentration is increased, the monomers aggregate to form micelles. The concentration at which micelles first begin to form is known as the critical micelle concentration (CMC). This concentration corresponds to the point where the surfactant first shows a stable low surface tension value [3].

Almost all surfactants being currently produced are chemically derived from petroleum. However, these synthetic surfactants are usually toxic themselves and hardly degraded by microorganisms. They are, therefore, a potential source of pollution and damage to the environment. These hazards associated with synthetic emulsifiers have, in recent years, drawn much attention to the microbial production of surfactants (biosurfactants) [4].

Biosurfactants are derived from living organisms, mainly microorganisms, and have attracted much attention because of advantageous characteristics such as structural diversity, low toxicity, higher biodegradability, better environmental compatibility, higher substrate selectivity, biodegradability, and lower CMC. These properties have led to several biosurfactant applications in the food, cosmetic and pharmaceutical industries [5,6].

The most commonly isolated biosurfactants are glycolipids and lipopeptides. They include rhamnolipids released by *Pseudomonas aeruginosa* [7], sophorolipids from *Candida* species [8], as well as surfactin and iturin produced by *Bacillus subtilis* strains [9]. The production yields of these biosurfactants are relatively high (2–10 g/l) and they reduce the surface tension of water to values bellow 30 mN/m [10]. Furthermore, *Candida lipolytica* UCP 0988 was found to produce 4.5 g/l of biosurfactant and this polymeric structure was capable of lowering the surface tension of water values around 32 mN/m [11].

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Several biosurfactants exhibit antibacterial, antifungal and antiviral activities, which make them relevant molecules for applications in combating many diseases and infections [12]. Biosurfactants with known antimicrobial activity include surfactin and iturin produced by *B. subtilis* strains [9], mannosylerythritol lipids from *Candida antarctica* [13], rhamnolipids from *P. aeruginosa* [14] and biosurfactants isolated from *Streptococcus thermophilus* A and *Lactococcus lactis* 53 [15–17]. Another valuable application of biosurfactants is their use as anti-adhesive agents against pathogens. Adsorption of biosurfactants to a substratum surface modifies its hydrophobicity, interfering in the microbial adhesion and desorption processes [18]; in that sense, the release of biosurfactants by probiotic bacteria in vivo can be considered as a defence weapon against other colonizing strains in the urogenital and gastrointestinal tracts [19]. Biosurfactants produced by *Lactobacillus paracasei* have been shown to reduce adhesion of pathogenic and non-pathogenic microorganisms [20,21].

Considering the lack of studies with yeasts biosurfactants for medical purposes and the attractive characteristics showed by the biosurfactant produced by the *C. lipolytica* strain UCP 0988, the aim of this work was to study the antimicrobial and anti-adhesive properties of this biosurfactant against pathogenic and nonpathogenic microorganisms. Results gathered in the current work showed the potential of the biosurfactants in this field of application. However, their use still remains limited, possibly due to their comparatively high production costs, as well as scant information on their toxicity towards human systems.

2. Materials and methods

2.1. Microorganisms and growth conditions

The microorganism *Candida lipolytica* UCP 0988 was kindly supplied from the Culture Collection of Nucleus of Research in Environmental Sciences, Catholic University of Pernambuco, Recife-PE, Brazil, registered in the World Federation of Culture Collection (WFCC). The microorganism was maintained in an anamorphic 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.

Several strains that commonly colonize prostheses and medical devices were used to test the antimicrobial and anti-adhesive properties of the biosurfactant. *Lactobacillus casei* 36, *Lactobacillus casei* 72, *Lactobacillus reuteri* 104R and *Lactobacillus reuteri* ML1 were cultured in MRS broth; *Streptococcus mutans*, *Streptococcus mutans* NS, *Streptococcus mutans* HG985, *Streptococcus oralis* J22, *Streptococcus sanguis* 12, *Rothia dentocariosa* and *Streptococcus salivarius* were cultured in Todd-Hewitt Broth; *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus agalactiae* and *Streptococcus pyogenes* were cultured in Trypticase Soy Broth (TSB); *Candida albicans* and *Candida tropicalis* were grown in yeast mould broth (YMB) (all media were obtained from Oxoid). All the strains were grown at 37 °C, with the exception of *C. albicans* and *C. tropicalis* (30 °C). Strains were stored at −80 °C in the appropriate medium containing 15% (v/v) glycerol solution until they were used. Whenever required, frozen stocks were streaked on agar plates and incubated overnight at the optimum growing temperature for each strain for further culturing. Working stock cultures were kept at 4 °C for up to 2 weeks [20].

2.2. 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% $MgSO_4{\cdot}7H_2O$. The medium was supplemented with soybean oil refinery residue and glutamic acid [1]. The refinery residue was obtained from ASA Indústria e Comércio LTDA (Recife-PE, Brazil). The composition of the refinery residue was previously described [22].

The inoculums were prepared in an Erlenmeyer flask with a capacity of 250 ml containing 50 ml of YMB and were inoculated using a microbial loop, incubated in an orbital shaker at 150 rpm and 28 °C for 24 h. The pH of the culture medium was adjusted to 5.7 by addition 1 M NaOH solution or 1 M HCl solution. All fermentations were conducted in 250 ml Erlenmeyer flasks containing 50 ml of the production medium. Immediately after inoculation of 5% of 10^8 cells/ml, the flasks were incubated for 72 h at 28 °C in an orbital shaker at 150 rpm.

2.3. Isolation of biosurfactant

The 72 h culture was filtered through 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 separator funnel at 28 °C [23].

2.4. Surface activity

Surface tension was determined on cell-free broth obtained by centrifuging the cultures at 5000 × *g* for 20 min with a Tensiometer model Sigma 70 (KSV Instruments LTD, Finland) using the Du Nouy ring method at room temperature.

2.5. Critical micelle concentration (CMC)

The surface tension was measured by the ring method using a DuNouy Tensiometer model Sigma 70 (KSV Instruments LTD, Finland) at room temperature. The concentration at which micelles began to form was represented as the 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. Antimicrobial assays

The antimicrobial activity of the crude biosurfactant produced by *C. lipolytica* UCP 0988 against several microbial strains was determined by the microdilution method in 96-well flat-bottom plastic tissue culture plates (Greiner Bio-One GmbH, Frickenhausen, Germany) [20].

For each strain, appropriate medium and temperature were used (as previously described); briefly, 125 μl of sterile, double-strength culture medium were placed into the first column of the 96-well microplate and 125 μl of sterile, single-strength culture medium in the remaining wells. Subsequently, 125 μl of biosurfactant solution in phosphate buffer saline at a 100 mg/ml concentration (PBS: 10 mM KH_2PO_4/K_2HPO_4 and 150 mM NaCl with pH adjusted to 7.0) (100 mg/ml) were added to the first column of the microplate and mixed with the medium; this results in a biosurfactant concentration of 50 mg/ml; serially, 125 μl were transferred to the subsequent wells, discarding 125 μl of the mixture in the tenth column, so that the final volume for each well was 125 μl. This process results in twofold serial dilutions of the biosurfactant in the first 10 columns (50–0.09 mg/ml). Columns 11 and 12 did not contain biosurfactant and served as negative and growth controls, respectively. All the wells (except for the 11th column) were inoculated with 2.5 μl of an overnight culture at the defined optimum conditions, diluted to 10^8 cfu/ml. Microplates were covered and incubated for 48 h under the appropriate growth

conditions for each microorganism. Triplicate assays were performed for all biosurfactant concentrations used for each strain.

After 48 h of incubation, the absorbance at 600 nm was determined for each well. The growth inhibition percentages at different biosurfactant concentrations for each microorganism were calculated as (Eq. (1)):

$$\%\,\text{Growth inhibition}_c = \left[1 - \left(\frac{A_c}{A_0}\right)\right] \times 100 \quad (1)$$

where A_c represents the absorbance of the well with a biosurfactant concentration c and A_0 the absorbance of the control well (without biosurfactant) [20].

2.7. Anti-adhesion assays

The anti-adhesive activity of the crude biosurfactant isolated from *C. lipolytica* UCP 0988 against several microbial strains was quantified according to the procedure described by Heinemann et al. [24]. Briefly, the wells of a sterile 96-well flat-bottom polystyrene tissue culture plate (Greiner Bio-One GmbH) were filled with 200 μl of the crude biosurfactant. Several biosurfactant concentrations were tested ranging from 3 to 50 mg/ml. The plate was incubated for 18 h at 4 °C and subsequently washed twice with PBS. Control wells contained PBS buffer only. An aliquot of 200 μl of a washed bacterial or yeast suspension (10^8 cfu/ml) was added and incubated in the wells for 4 h at 4 °C. Unattached microorganisms were removed by washing the wells three times with PBS. The adherent microorganisms were fixed with 200 μl of methanol (99% purity) per well, and after 15 min, the plates were emptied and left to dry. Then the plates were stained for 5 min with 200 μl of 2% crystal violet used for Gram staining per well. Excess stain was rinsed out by placing the plate under running tap water. Subsequently, the plates were air dried, the dye bound to the adherent microorganisms was resolubilized with 200 μl of 33% (v/v) glacial acetic acid per well, and the absorbance of each well was measured at 595 nm. The microbial inhibition percentages at different biosurfactant concentrations for each microorganism were calculated as (Eq. (2)):

$$\%\,\text{Microbial inhibition}_c = \left[1 - \left(\frac{A_c}{A_0}\right)\right] \times 100 \quad (2)$$

where A_c represents the absorbance of the well with a biosurfactant concentration c and A_0 the absorbance of the control well. The microtitre-plate anti-adhesion assay estimates the percentage of microbial adhesion reduction in relation to the control wells, which were set at 0% to indicate the absence of biosurfactant and therefore of its anti-adhesion properties. In contrast, negative percentage results indicate the percentage increase in microbial adhesion at a given surfactant concentration in relation to the control. The microtitre-plate anti-adhesion assay allows the estimation of the crude biosurfactant concentrations that are effective in decreasing adhesion of the microorganisms studied.

3. Results and discussion

3.1. Yield, surface tension and critical micelle concentration of the biosurfactant

The yield of the crude biosurfactant produced by *C. lipolytica* UCP 0988 grown on industrial refinery residue after 72 h was 8 g/l. The biosurfactant was able to reduce the medium surface tension from 50.0 mN/m to 25.0 mN/m. On the other hand, *Candida bombicola* grown on glucose and arachidonic acid produced sophorolipids up to 1.44 g/l after 96 h [25], while *C. lipolytica* grown on industrial refinery residue produced 4.5 g/l of biosurfactant after 144 h

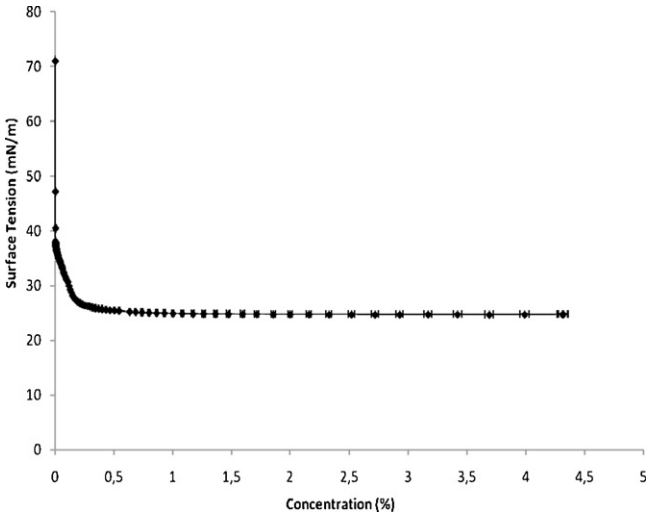

Fig. 1. Surface tension versus concentration of the isolated biosurfactant produced by *Candida lipolytica* UCP 0988 grown in medium supplemented with soybean oil refinery residue and glutamic acid.

[11]. The biosurfactants produced by yeasts described in the literature also show low surface tension values as the biosurfactant from *C. lipolytica* (32 mN/m) [11], from *Candida glabrata* (31 mN/m) [26], from *C. antarctica* (35 mN/m) [27] and from *Yarrowia lipolytica* (50 mN/m) [28].

The CMC is a widely used index to evaluate surface activity. By definition, the CMC is the surfactant concentration of surfactant above which micelles are spontaneously formed. Until the CMC is reached a decrease in the surface tension will be observed. However, upon reaching the CMC, any further increase in the surfactant concentration will only increase the number of micelles and no alteration in the surface tension will be observed [29].

The relationship between surface tension and concentration of the isolated biosurfactant solution was determined in an automatic tensiometer (Fig. 1). The biosurfactant exhibited excellent surface tension reducing activity. The surface tension of water of 71 mN/m decreased to 25.0 mN/m by increasing the solution concentration up to 3 mg/l. Further increase in the concentration of the biosurfactant solution did not reduce the surface tension of water, indicating that the CMC was reached at this concentration.

The biosurfactant produced by *C. lipolytica* showed CMC values of 2.5% [30], while the biosurfactant from *C. antarctica* showed a concentration of 0.6% mg/l at the CMC [27]. The biosurfactant produced by *Lactobacillus paracasei* exhibited a minimum surface tension value of 41.8 mN/m for a concentration of 50 mg/ml [21].

3.2. Antimicrobial activity

Several biosurfactants which exhibit antimicrobial activity against various microorganisms have been previously described. They include surfactin and iturin produced by *Bacillus subtilis* strains [9], rhamnolipids from *Pseudomonas* species [14,31], mannosylerythritol lipids from *C. antarctica* [13] and biosurfactants produced by some fungi [32].

The antimicrobial activity of the crude biosurfactant isolated from *Candida lipolytica* UCP 0988 was determined by measuring the growth inhibition percentages obtained for several microorganisms (Table 1).

The biosurfactant was effective against the microorganisms tested, albeit to different degrees. The highest anti-adhesive percentages were obtained for a biosurfactant concentration of 12 mg/l or 4×CMC. Non-pathogenic species associated with the oral cavity

Table 1
Percentages of growth inhibition obtained with the biosurfactant isolated from *Candida lipolytica* UCP 0988 at different concentrations (mg/l). Results are expressed as means ± standard deviations of values obtained from triplicate experiments.

Microorganism	Growth inhibition (%)				
	Biosurfactant (mg/l)				
	0.75	1.5	3	6	12
Lactobacillus casei	4.5 ± 0.02	9.1 ± 0.07	15 ± 0.02	27.2 ± 0.08	28.4 ± 0.05
Lactobacillus casei 72	4.5 ± 0.03	14.2 ± 0.08	18.1 ± 0.06	32.9 ± 0.06	33.7 ± 0.03
Lactobacillus reuteri 104R	5.9 ± 0.07	9.7 ± 0.01	15.5 ± 0.01	24.4 ± 0.05	25.4 ± 0.08
Lactobacillus reuteri ML1	8.2 ± 0.06	11.3 ± 0.02	16.3 ± 0.02	31.1 ± 0.02	32.1 ± 0.02
Escherichia coli	0	0	3.0 ± 0.01	5.0 ± 0.01	5.0 ± 0.01
Streptococcus agalactiae	0	10.9 ± 0.03	35.3 ± 0.03	35.8 ± 0.02	35.5 ± 0.02
Streptococcus mutans NS	15.6 ± 0.07	20.1 ± 0.04	23.8 ± 0.13	46.0 ± 0.1	46.4 ± 0.01
Streptococcus sanguis 12	13.7 ± 0.07	21.5 ± 0.04	31.7 ± 0.05	48.1 ± 0.08	48.0 ± 0.01
Streptococcus mutans	22.5 ± 0.02	34.8 ± 0.01	44.6 ± 0.01	58.3 ± 0.01	58.0 ± 0.06
Streptococcus oralis J22	12.8 ± 0.04	13.2 ± 0.03	14.2 ± 0.03	18.7 ± 0.08	62.8 ± 0.06
Streptococcus mutans HG985	41.8 ± 0.02	43.1 ± 0.01	55.6 ± 0.04	64.6 ± 0.03	64.9 ± 0.01
Pseudomonas aeruginosa	0	7.9 ± 0.02	10 ± 0.01	11.6 ± 0.01	16.5 ± 0.04
Staphylococcus aureus	0	0	1.57 ± 0.06	3.15 ± 0.06	15.1 ± 0.03
Staphylococcus epidermidis	10.1 ± 0.01	12.9 ± 0.07	14.9 ± 0.07	18.1 ± 0.01	18.0 ± 0.06
Candida albicans	0	0	3.1 ± 0.03	5.95 ± 0.01	6.0 ± 0.02

of *Streptococcus* were used (*S. mutans* HG – 64.9%; *S. oralis* J22 – 62.8%; *S. mutans* – 58%; *S. sanguis* 12 – 48%; *S. mutans* NS – 46%). On the other hand, the biosurfactant did not show an effective antimicrobial activity against the *Lactobacillus* strains studied. It inhibited only 32.1% of the growth of *L. reuteri* ML1 at the maximum concentration tested (12 mg/l).

The growth of the other microorganisms tested was poorly inhibited. Percentages of 5%, 5%, 15%, 16% and 18% were observed for *C. albicans, E. coli*, *S. aureus, P. aeruginosa* and *S. epidermidis*, respectively.

3.3. Anti-adhesive activity

Involvement of biosurfactants in microbial adhesion and desorption has been widely described, and adsorption of biosurfactants to solid surfaces might constitute an effective strategy to reduce microbial adhesion and combating colonization by pathogenic microorganisms, not only in the biomedical field, but also in other areas, such as the food industry [16,33–35].

In addition to the antimicrobial properties, the anti-adhesive activity of the biosurfactant was evaluated against a variety of bacterial and fungal strains. The biosurfactant showed anti-adhesive activity against most of the microorganisms tested, but the anti-adhesive effect depends on the concentration and the micro-organism tested (Table 2).

The crude biosurfactant showed anti-adhesive activity against most of the microorganisms tested from the minimum concentration used (0.75 mg/l). The anti-adhesive property was proportional to the concentration of the biosurfactant. For the microorganisms of the *Lactobacillus* anti-adhesive values around 81% were observed at the minor concentration tested (0.75 mg/l). The major anti-adhesive specificity was observed against *L. casei* with values of 91% and 99% with the minimum concentration used. Low inhibitions were observed for *S. epidermidis* and *E. coli*, with values of 27% and 21%, respectively, at the maximum biosurfactant concentration. For the other microorganisms, the anti-adhesive activity was above 45%.

Gudina et al. [21] observed an anti-adhesive activity for the biosurfactant from *Lactobacillus paracasei* against several pathogenic microorganisms such as *S. aureus*, *S. epidermidis* and *S. agalactiae*. However, this biosurfactant showed low anti-adhesive activity against *E. coli*, *C. albicans* and *P. aeruginosa*, in contrast with the antimicrobial activity exhibited against these strains at the same biosurfactant concentrations.

The use and potential commercial applications of biosurfactants in the medical field has increased considerably in the last

Table 2
Anti-adhesive properties of crude biosurfactant isolated from *Candida lipolytica* UCP 0988. Negative controls were set at 0% to indicate the absence of biosurfactant. Positive percentages indicate the reductions in microbial adhesion when compared to the control. Results are expressed as means ± standard deviation of results from triplicate experiments.

Microorganism	Antiadhesive Activity (%)					
	Biosurfactant (mg/l)					
	0.75	1.5	3	6	12	Control (PBS)
Lactobacillus casei	91 ± 0.1	91 ± 0.1	99 ± 0.1	99 ± 0.1	99 ± 0.1	0
Lactobacillus casei 72	81 ± 0.0	87 ± 0.0	89 ± 0.0	91 ± 0.0	95 ± 0.0	0
Lactobacillus reuteri 104R	84 ± 0.2	93 ± 0.2	94 ± 0.2	95 ± 0.2	97 ± 0.2	0
Lactobacillus reuteri ML1	81 ± 0.0	82 ± 0.0	84 ± 0.0	87 ± 0.0	89 ± 0.0	0
Escherichia coli	8 ± 0.1	17 ± 0.1	18 ± 0.1	25 ± 0.1	27 ± 0.1	0
Streptococcus agalactiae	80 ± 0.0	81 ± 0.0	81 ± 0.0	84 ± 0.0	96 ± 0.0	0
Streptococcus mutans NS	91 ± 0.1	95 ± 0.1	96 ± 0.1	98 ± 0.1	99 ± 0.1	0
Streptococcus sanguis 12	61 ± 0.0	62 ± 0.0	68 ± 0.0	70 ± 0.0	77 ± 0.0	0
Streptococcus mutans	76 ± 0.1	84 ± 0.1	85 ± 0.1	88 ± 0.1	97 ± 0.1	0
Streptococcus oralis J22	73 ± 0.0	85 ± 0.0	87 ± 0.0	89 ± 0.0	90 ± 0.0	0
Streptococcus mutans HG985	76 ± 0.1	76 ± 0.1	81 ± 0.1	83 ± 0.1	85 ± 0.1	0
Pseudomonas aeruginosa	13 ± 0.0	26 ± 0.0	33 ± 0.0	41 ± 0.0	49 ± 0.0	0
Staphylococcus aureus	88 ± 0.0	91 ± 0.0	92 ± 0.0	97 ± 0.0	98 ± 0.0	0
Staphylococcus epidermidis	2 ± 0.0	5 ± 0.0	6 ± 0.0	16 ± 0.0	21 ± 0.0	0
Candida albicans	8 ± 0.0	8 ± 0.0	16 ± 0.0	36 ± 0.0	51 ± 0.0	0

years. Their antimicrobial and anti-adhesive properties make them relevant molecules for use in combating many diseases and infections and as therapeutic agents [36]. Falagas and Makris [35] have proposed the application of biosurfactants isolated from probiotic bacteria to patient care equipments (such as catheters and other medical insertional devices) in hospitals, with the aim of decreasing colonization by microorganisms responsible for nosocomial infections.

4. Conclusions

In conclusion, in this work we have demonstrated the antimicrobial and anti-adhesive properties of the crude biosurfactant isolated from *C. lipolytica* UCP 0988 against several pathogenic and nonpathogenic microorganisms, including bacteria, yeasts and filamentous fungi. The results obtained suggest the possible use of this biosurfactant as an alternative antimicrobial agent in the medical field for applications against microorganisms responsible for diseases and infections, making it a suitable alternative to conventional antibiotics.

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