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Assessment of toxicity of a biosurfactant from *Candida sphaerica* UCP 0995 cultivated with industrial residues in a bioreactor

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Abstract

Background: The aim of the present study was to propose a low-cost method for the production of a biosurfactant by the yeast *Candida sphaerica* and assess its toxicity and phytotoxicity. The medium was formulated with distilled water supplemented with residue from a soy oil refinery (5%) and corn steep liquor (2.5%) as substrates. These two products were the sources of carbon and nitrogen as well as mineral elements to encourage the growth of the microorganism and production of a biosurfactant.
Results: The isolated biosurfactant yield was 6.364 g/l. The biosurfactant exhibited an excellent ability to reduce surface tension (26 mN/m) and demonstrated no toxicity against seeds of *Brassica oleracea*, *Chicoria intybus* and *Solanum gilo* or the micro crustacean *Artemia salina* employed as a bioindicator. The biosurfactant exhibited no antimicrobial activity against the fungi and bacteria tested.
Conclusions: The promising results obtained in this study indicate the feasibility of producing biosurfactants from powerful non-toxic organic residues and their application in the bioremediation of contaminated soil and water.

Keywords: biosurfactant, bioreactor, *Candida sphaerica,* industrial residues, toxicity.

INTRODUCTION

Most commercially available surfactants are synthesized from petroleum derivatives. However, new environmental control legislation has encouraged the development of natural surfactants, such as those of a microbiological origin (biosurfactants) (Banat et al. 2010; Rufino et al. 2011). Biosurfactants have received growing interest in recent decades due to their advantages over synthetic products, such as biodegradability, low toxicity, production from renewable sources and functionality under extreme pH and temperature conditions (Singh et al. 2007; Calvo et al. 2009).

However, high production costs make biosurfactants less competitive than synthetic products. The fermentation process is the key to reducing such costs with the use of alternative substrates, which represent approximately 30% of the overall cost. In this context, industrial residues have emerged as promising low-cost substrates (Mukherjee et al. 2006). Agro-industrial substrates with high carbohydrate and lipid contents generally met the carbon needs for the production of biosurfactants (Mercade and Manresa, 1994; Sarubbo et al. 2006; Sarubbo et al. 2007; Coimbra et al. 2009; Gusmão et al. 2010).

The use of biosurfactants also depends on their properties. The absence of toxicity is of fundamental importance for applications in the environmental realm. Ecotoxicity bioassays are analytical methods that allow characterizing the toxicity of chemical substances. The exposure of living microorganisms employed as bioindicators to these substances constitutes a valuable environmental analysis tool (Fletcher, 1991).

A number of biosurfactants exhibit antimicrobial activity, such as those produced by species of *Bacillus*. Surfactin, which is one of the most well-known biosurfactants, and lutrin, which is a lipopeptide produced by *B. subtilis*, have antifungal properties (Nitschke and Pastore, 2002). The biosurfactant rufisan, obtained by Rufino et al. (2011) in a low-cost medium, has demonstrated antimicrobial activity against several species of microorganisms.

The aim of the present study was to propose a low-cost method for the production of a biosurfactant by the UCP 0995 strain of the yeast *C. sphaerica* and characterize its toxicity.

MATERIALS AND METHODS

Microorganism

Candida sphaerica (UCP 995), a yeast isolated from mangrove sediment collected in the municipality of Rio Formoso in the state of Pernambuco, Brazil and deposited in the Culture Bank of the Center for Research in Environmental Science of the Universidade Católica de Pernambuco, was used as the microorganism for the production of biosurfactant. The culture was kept at 5ºC on a yeast mold agar (YMA) medium.

Characterization of microorganism

Cells from *C. sphaerica* UCP 0995 were cultivated in a yeast extract-peptone-dextrose medium for 24 hrs and DNA was extracted using the phenol-chloroform method. The genetic material was submitted to amplification by polymerase chain reaction (PCR) using the (GTG) primer, based on the method described by Silva-Filho et al. (2005). The amplified fragments were separated on 1.2% agarose gel using the tris-acetate-ETDA buffer.

Reagents and substrates

All reagents used were of analytical grade. Culture media were obtained from Difco, USA. Two industrial residues were used as substrates for biosurfactant production. An industrial residue from a soybean oil refinery (kindly donated by ASA LTDA, Recife, PE, Brazil) was used as the insoluble substrate. Corn steep liquor, which is a byproduct of corn manufacturing industry (kindly donated by Corn Products do Brasil, Cabo de Santo Agostinho, PE, Brazil), was used as the soluble substrate. The soybean oil refinery residue constituted the main carbon source and corn steep liquor was the nitrogen source. Both residues also provided other important nutrients for the metabolism of the microorganism (Sobrinho et al. 2008).

Culture media

YMA medium was used for maintaining *C. sphaerica* UCP 0995 and had the following composition: yeast extract (0.3%), malt extract (0.3%), D-glucose (1.0%), tryptone (0.5%), agar (5.0%) and distilled water q.s.p. (100 ml). The components were solubilized and sterilized in an autoclave at 121ºC for 20 min. When the agar was excluded, the solution constituted the growth medium for the inoculum - yeast mold broth (YMB). Transfers were performed monthly to maintain cell viability. Biosurfactant production was performed in distilled water supplemented with soybean oil refinery residue (5%) and corn steep liquor (2.5%) as substrates, following the method described by Sobrinho et al. (2008).

Preparation of inoculums

The inoculum was standardized by transferring a sample of *C. sphaerica* UCP 0995 to test tubes containing the YMA medium. After growth for 72 hrs at 28ºC, the cultures were transferred to 250-ml Erlenmeyer flasks containing 50 ml of the YMB medium. The inoculum (1%, v/v) was introduced in the amount of 10^4 cells/ml and incubated under orbital shaking at 150 rpm and 28ºC for 24 hrs.

Biosurfactant production

Fermentation for biosurfactant production was performed by amplifying the production scale in a 5-L bioreactor (Bioflo 2000, USA). The bioreactor was operated with no control of pH or temperature. Initial pH was 5.3 and agitation was maintained at 5.7 rpm and 27ºC. Samples were withdrawn every 24 hrs until 144 hrs of culture and used for the determination of biomass, pH, surface tension and biosurfactant yield. All analyses were performed in triplicate.

Determination of biomass

Growth was followed through the determination of biomass (dry weight). Samples (50 ml) were centrifuged at 2000 g for 20 min. The biomass was washed in distilled water in a graduated centrifuge tube. Following shaking and further centrifugation, the upper phase was discarded and the cell pellet was dried in an oven at 105ºC for 24 hrs and weighed.

Isolation of biosurfactant

After 144 hrs of culture *C. sphaerica* UCP 0995 in the bioreactor, the broth was centrifuged at 2000 g for 20 min for cell removal and submitted to the extraction process. The pH was adjusted to 2 with 6.0 M of HCl solution and precipitated with 2 volumes of methanol. After resting for 24 hrs at -15ºC, the samples were centrifuged at 4000 g and 28ºC for 30 min, washed twice with cooled methanol, dried at 37ºC for 48 hrs and maintained in the desiccator until reaching a constant weight. The yield of the isolated product was calculated as g/l. All analyses were performed in triplicate.

Determination of surface tension

Surface tension was measured in the cell-free broth using an automatic tensiometer (Sigma 70, KSV Ltd., Finland) and the Du Nouy ring. Stabilization was allowed to occur until the standard deviation of successive measurements was less than 0.4 mN/m. Each result was the mean of 10 determinations following the stabilization of the reading.

Determination of antimicrobial activity of biosurfactant in Petri dishes

The evaluation of the antimicrobial activity of the biosurfactant was performed with the bacteria *Staphylococcus aureus* UFPEDA02*, Escherichia coli* UFPEDA 224*, Pseudomonas aeruginosa* UFPEDA 416*, Enterococcus fecalis* UFPEDA 138*, Bacillus subtilis* UFPEDA 86 and *Klebsiella pneumoniae* UFPEDA 396 from the microorganism bank of the Department of Antibiotics, Universidade Federal de Pernambuco (Brazil) and the yeasts *C. sphaerica* UCP 0995, *C. glabrata* UCP 1002 and *C. lipolytica* UCP 0988 from the microorganism bank of the Culture Bank of the Center for Research in Environmental Science (Brazil) using the disk diffusion method in the Mueller-Hinton agar (Oxoid) culture medium (Barry and Thornsberry, 1991).

The bacterial suspension was obtained from a broth culture, incubated for 24 hrs at 37ºC and diluted until turbidity of the solution corresponded to 0.5 on the MacFarland scale. The reading was performed in a spectrophotometer at 600 nm for a final bacterial concentration of 10^8 CFU/ml. Standardized suspensions (2 ml) containing the microorganism were inoculated in Petri dishes (90 mm in diameter) containing the Mueller-Hinton medium. Preliminary screening for the determination of bacterial susceptibility to the extracts was performed using Whatman Nº 3 paper disks measuring 10 mm in diameter impregnated with 20 µl of the extracted biosurfactant at concentrations of 200, 400 and 600 mg/l placed on the surface of the solid medium and incubated at 37ºC for 24 hrs. The concentrations

used were based on the biosurfactant critical micelle concentration (800 mg/l), as determined by Sobrinho et al. (2008).

The diameter of the inhibition halo was measured in millimeters using a caliper. Readings of the inhibition halos were performed for 48 hrs. All analyses were performed in triplicate.

Determination of antimicrobial activity using microdilution method

The determination of antimicrobial activity using the microdilution method was performed in Mueller-Hinton broth in 96-well ELISA plates with a U-shaped bottom to allow better visualization of bacterial growth, based on the methodology described by Eloff (1998).

The biosurfactant was solubilized in dimethyl sulfoxide (DMSO). Stock solutions of the solubilized biosurfactant were initially prepared at a concentration of 2.0 µg/ml. When applied on the plates, the solutions were diluted twice at the highest concentration tested (500 µg/ml). Dilutions were then made at a proportion of 1:2 for the remaining wells. The maximal concentration of DMSO was 2.5%. Controls were prepared using solvent to determine the possible activity against the bacteria tested. The positive control was the culture medium and inoculum and the negative control was the culture medium alone. Ampicillin was used as the antimicrobial control.

The bacteria used in the microdilution test were stored at -20ºC. The microorganisms tested were *E. faecalis* UFPEDA 138, *B. subtilis* UFPEDA 86, *P. aeruginosa* UFPEDA 416 and *K. pneumonia* UFPEDA 396. The species were placed in tryptic soy broth and incubated at 37ºC for 24 hrs in a standardized suspension using the MacFarland scale to obtain a concentration of 10^8 CFU/ml. The reading was performed in a spectrophotometer.

The determination of the antimicrobial activity of the biosurfactant produced by *C. sphaerica* was performed using the microdilution method proposed by the Clinical and Laboratory Standards Institute (2003). For such, sterilized 96-well microplates with a U-shaped bottom were used (TPP®, USA). Aliquots of 5 µL of the Mueller-Hinton culture medium, 50 µl of the biosurfactant at different concentrations (200, 400 and 600 mg/l) and 5 µl of the inoculum of each species tested were placed in each well. Immediately following micro-pipetting, the plates were covered and incubated at 37ºC for 24 hrs, without shaking. Following agitation of the wells to re-suspend the adhered cells with a multi-channel pipette, readings were performed at 600 nm in a microplate reader (LGC model). A negative control (culture medium and 2.5% DMSO), growth control (culture medium, 2.5% DMSO and inoculum) and positive control (water, culture medium, inoculum, 2.5% DMSO and antibiotic) were used for each microplate.

Assessment of toxicity using the germination test

The germination and root growth method suggested by Yerushalmi et al. (2003) was carried out using seeds from the cauliflower *Brassica oleracea*, the chicory *Cichorium intybus* and the scarlet eggplant *Solanum gilo.* Different concentrations of the isolated biosurfactant solution (200, 400 and 600 mg/l) as well as the cell-free broth (crude biosurfactant) and control solution (distilled water) were tested. The biosurfactant samples were placed in Petri dishes with 10 seeds from each species. The dishes were incubated for 120 hrs at 27ºC. The number of germinated seeds was then counted and the length of the roots was measured from the point of transition from the hypocotyl to the extremity of the root. The germination index (GI), which is one of the most commonly used ways to characterize the phytotoxicity of a compound, was calculated as follows:

%GI = (% of seed germination) x (% of root growth): 100

in which % of seed germination = (% of germination in the extract): (% of germination in the control) x 100; and % of root growth = (mean growth in the extract): (mean growth in the control) x 100. The analyses were performed in triplicate.

Assessment of toxicity using *Artemia salina*

The toxicity tests were performed with different concentrations of the isolated biosurfactant (200, 400 and 600 mg/l). Biosurfactant samples (20 ml) were used in 24 hrs assays with the micro crustacean *Artemia salina*. The bioassay was based only on the percentage of dead organisms in relation to the total number (10 larvae) in 5 ml of an aqueous solution containing synthetic marine salt (33.3 g/l) and 5 ml of the different concentrations of biosurfactant samples. The maximal volume of the test sample was 1.5 ml. After 24 hrs of incubation, the surviving organisms were quantified and the 50% lethal concentration (LC_{50}) of the samples was determined (Mc Laughlin et al. 1985). The analyses were performed in triplicate.

RESULTS AND DISCUSSION

Characterization of *Candida sphaerica* UCP 0995

The analysis of the microorganism strain was first carried out. The results of the taxonomic analysis demonstrated that the yeast *C. sphaerica* UCP 0995 was an anamorph of the yeast *Kluyveromyces lactis* (Figure 1).

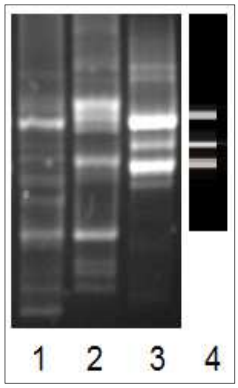

Fig. 1 Gel electrophoresis in 1.2% agarose displaying amplification pattern through PCR fingerprinting using the $(GTG)_5$ primer of the yeasts *S. cerevisiae* JP1 (column 1), *Dekkera bruxellensis* GDB248 (column 2) and the *C. sphaerica* isolate (column 3). The amplification profile for *Kluyveromyces lactis* CTC56498 was taken from Silva et al. (2006).

Kinetics of *Candida sphaerica* UCP 0995 growth and biosurfactant production

Figure 2 displays the kinetics of *C. sphaerica* UCP 0995 growth and production of the biosurfactant cultured in distilled water supplemented with 5.0% soybean oil refinery residue and 2.5% corn steep liquor in a bioreactor. Biosurfactant yield was 6.364 g/l after 144 hrs of culture. A recent study carried out by Sobrinho et al. (2008) with the same production medium using the shake-flask method reports a yield of 4.5 g/l after 144 hrs of culture. It is possible that aeration and agitation parameters favoured cell growth and a greater biosurfactant yield when the culture was performed in a bioreactor.

The exponential growth phase of *C. sphaerica* occurred at 48 hrs through to 96 of culturing, when maximal biomass production was observed (9.47 g/l). The greatest production of biosurfactant occurred in the stationary growth phase, which was evidenced by the reduction in surface tension of the culture medium from 55 to 27 mN/m. The initial pH of the medium was 5.3 and rose in the first 24 hrs to around 7.0, at which point, it remained unaltered through to the end of the culture process.

Studies carried out by Sarubbo et al. (2006) on biosurfactant production by *C. glabrata* using two industrial residues as substrates also demonstrated a reduction in surface tension in the stationary

growth phase. In contrast, Mulligan (2005) reports the production of surfactin by *B. subtilis* beginning in the exponential growth phase.

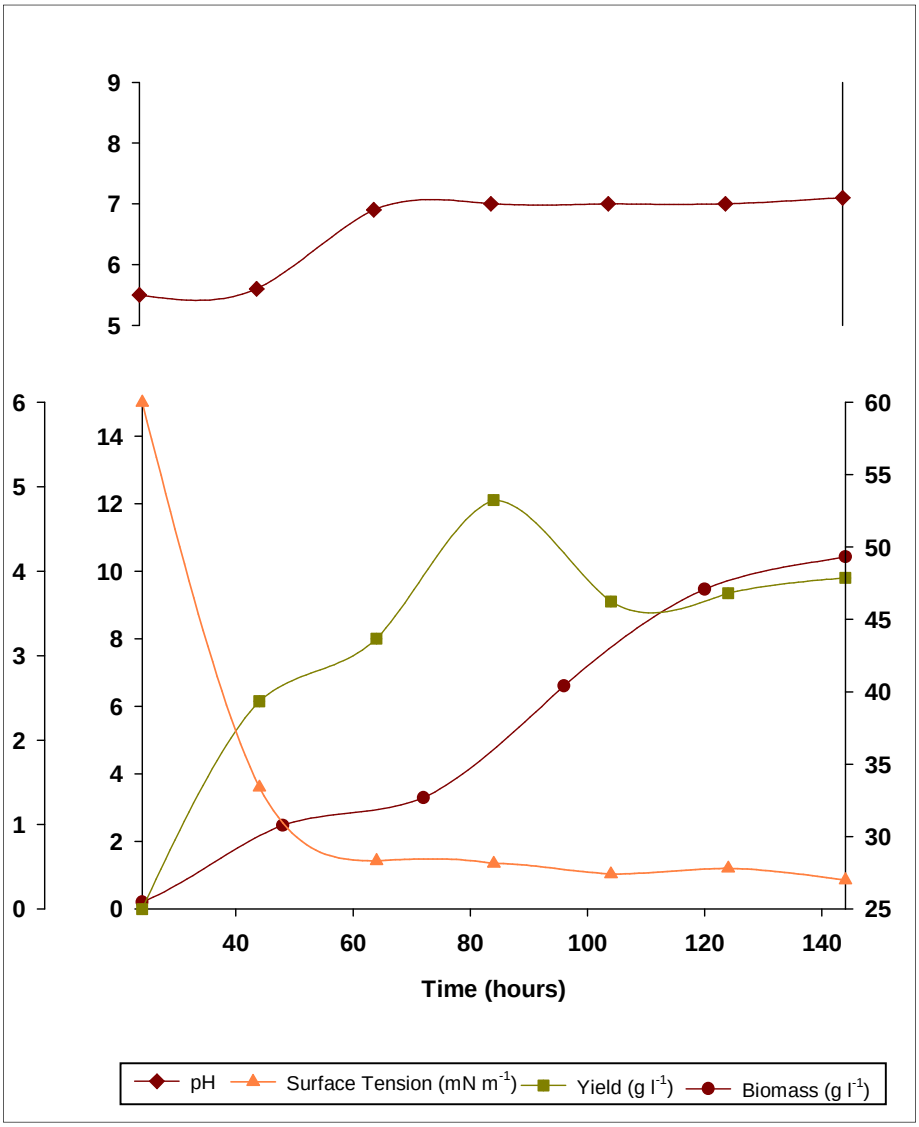

Fig. 2 Growth, pH, surface tension and yield of biosurfactant isolated from *C. sphaerica* cultured in a bioreactor in distilled water with 5.0% soybean oil refinery residue and 2.5% corn steep liquor.

According to the literature, bacterial biosurfactants are more effective at reducing surface tension. *P. aeruginosa* is the most often studied bacterium for the production of potent biosurfactants, which have demonstrated the ability to reduce surface tension to around 27 mN/m (Santa Anna et al. 2001; Gautam and Tyagi, 2006). However, biosurfactants produced by yeasts in recent decades have demonstrated the ability to reduce surface tension to around 25 mN/m (Rufino et al. 2007) and recent studies report values similar to those achieved with bacterial biosurfactants, such as the result obtained in the present study for the biosurfactant from *C. sphaerica* UCP 0995. Rufino et al. (2007) and Rufino et al. (2011) found that a biosurfactant produced by the yeast *C. lipolytica* UCP 0988 cultured in industrial soybean oil refinery residue as the substrate reduced the surface tension of the medium from 50 mN m^{-1} to 26 mN/m after 72 hrs of culture. In another study involving the use of cottonseed oil, glucose and yeast extract for the production of biosurfactant by *C. glabrata* UCP 1002, Luna et al. (2009) report a reduction in surface tension to 31 mN/m after 144 hrs of culture.

Biosurfactant yield by yeasts is compatible with that obtained with bacteria. Rufino et al. (2011) report a yield of 8 g/l after 72 hrs of fermentation with the lipopeptide rufisan. Luna et al. (2011) report a yield of 9 g/l for the biosurfactant lunasan produced by *C. sphaerica* on a low-cost substrate after 144 hrs of fermentation.

Phytotoxicity

In recent decades, a large number of publications have been dedicated to explaining the reasons why some seeds fail to germinate in particular concentrations of substrates. The germination test has been employed in phytotoxicity assays due to its low execution cost. According to Fletcher (1991), tests involving plants are based on seed germination, root growth and seedling growth and plants that are sensitive to toxic substances can be used as bioindicators. The literature reports that some surfactants have an inhibitory effect on plant growth (Stahlman et al. 1997).

Figure 3 displays the results of the phytotoxicity tests using the vegetable species *C. intybus, S. gilo* and *B. oleracea* regarding the action of different concentrations of the biosurfactant isolated from *C. sphaerica* UCP 0995.

The phytotoxicity test involving *C. intybus* (Figure 3a) indicated an inhibitory effect on germination of 20 to 40% with the increase in the concentration of the biosurfactant. In contrast, root growth was practically constant (90 to 100%) in relation to the control (distilled water), with a reduction in root growth observed at a concentration of 600 mg/l. Regarding the seeds that germinated, the concentration of 200 mg/l allowed 100% germination in comparison to the control.

The results obtained with the extracts of the biosurfactant for seeds from *S. gilo* (Figure 3b) revealed 100% germination at the concentration of 200 mg/l in comparison with the control, whereas no germination occurred at concentrations of 400 and 600 mg/l, demonstrating an inhibitory effect at these concentrations. Regarding the emission of secondary roots and the germination index, values of 71.4% were found for the biosurfactant at the concentration of 200 mg/l, whereas the seeds did not emit secondary roots at concentrations of 400 and 600 mg/l. The results indicate a germination index of 100% for seeds of *B. oleracea* with the biosurfactant at a concentration of 600 mg/l in relation to the control, corresponding to 60% of the seeds having budded or emitted roots. Moreover, good percentages of seed germination were achieved with concentrations of 400 and 600 mg/l in comparison to 200 mg/l. Thus, the biosurfactant is not toxic to this species at the concentrations tested, as demonstrated by the lack of an inhibitory effect (Figure 3c).

At a concentration of 600 mg/l, a small amount of root growth occurred in relation to the control, which exhibited growth of 16 cm. With the cell-free broth, there was no root growth or germination of any of the seeds. It is likely that other substances in the extract inhibited plant growth.

Studying the lettuce *Lactuva sativa* submitted to biosurfactant concentrations of 0.4% and 0.8%, Millioli et al. (2007) found a constant germination index of 40% to 45%, suggesting that low concentrations of rhamnolipids may contribute to the fertilization of the soil. On the other hand, concentrations of 1 and 1.5% were sufficient to inhibit the germination index by 70% and 80%, respectively.

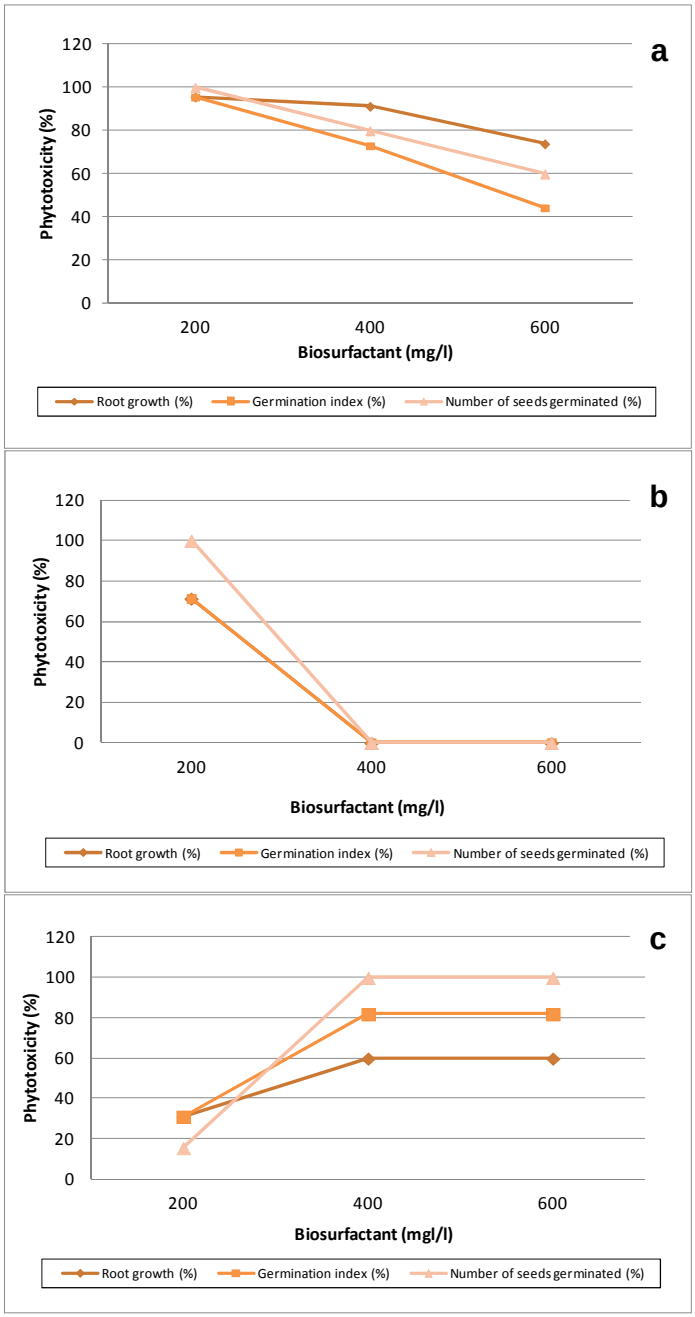

Fig. 3 Phytotoxicity of biosurfactant isolated from *C. sphaerica* UCP 0995 cultured in distilled water supplemented with 5.0% soybean oil refinery residue and 2.5% corn steep liquor regarding *C. intybus* (a), *S. gilo* (b) and *B. oleracea* (c).

In phytotoxicity tests with materials resulting from the biodegradation of diesel oil by fungal species, Souza et al. (2005) found that the residual liquid of the degradation of the oil achieved a germination index of 57%. Silva et al. (2010) performed phytotoxicity tests on *B. oleracea* using concentrations of 175, 350, 520 and 700 mg/l of a biosurfactant produced by *P. aeruginosa* UCP 0992 cultured in glycerol as the substrate. The results demonstrated no inhibitory effect regarding seed germination, with a germination index of 86%, indicating innocuousness with regard to this vegetal species.

Rufino et al. (2011) tested the phytotoxicity of the biosurfactant rufisan produced in a low-cost medium against *B. oleracea* and found a germination index of 136%. The biosurfactant did not exhibit inhibitory effects on the seeds or the growth of primary roots at the concentrations analyzed.

Antimicrobial activity

A number of biosurfactants exhibit antimicrobial activity, such as rhamnolipids produced by *P. aeruginosa* and lipopetides from *B. subtilis* (Lang and Philip, 1998).

In the present study, the biosurfactant produced by *C. sphaerica* UCP 0995 demonstrated no antimicrobial activity against the three yeasts of the genus *Candida* or the bacteria *E. coli, P. aeruginosa, E. fecalis* and *B. subtilis* at the concentrations tested. The biosurfactant exhibited bacteriostatic activity with *S. aureus* and *K. pneumoniae*. The bacterium *K. pneumoniae* did not exhibit an inhibition halo at concentrations of 400 and 600 mg/l, although bacteriostatic activity was observed in the presence of the biosurfactant solution at a concentration of 200 mg/l. In tests with *S. aureus,* no bacterial growth inhibition halos occurred, although a discrete bacteriostatic activity was observed when the bacterium was submitted to the higher concentration of the biosurfactant.

As a bactericide, the biosurfactant exhibited no effect against the microorganisms tested, thereby demonstrating its potential as a coadjuvant for application in bioremediation processes in contaminated soil and water. Considering the importance of the confirmation of the bactericidal activity of biosurfactants evidenced in some species of bacteria, microdilution tests (ELISA) were carried out, the results of which confirmed the absence of antimicrobial activity against both the fungi and bacteria tested.

For a biosurfactant produced by *Bacillus* sp. cultivated in sucrose as the carbon source, Bueno et al. (2010) found an absence of inhibition halos, indicating a lack of antibacterial and antifungal activity against the microorganisms tested, but the author found slight bacteriostatic activity. In contrast, Luna et al. (2011) carried out a study with a biosurfactant produced by *C. sphaerica* cultivated using soybean oil refinery residue and macerated corn and found antimicrobial activity against different species of fungi and bacteria, suggesting the use of this biomolecule as an alternative antimicrobial agent in the medical field.

Rufino et al. (2011) also tested the antimicrobial activity of a pure biosurfactant isolated from *C. lipolytica* UCP 0988 cultivated in soybean oil refinery residue, which was determined by measuring the percentage of growth inhibition toward different bacteria, fungi and yeasts. The biosurfactant exhibited antimicrobial activity against most of the microorganisms tested, with the exception of strains of Lactobacillus, which also suggests the possible use of this biosurfactant as an antimicrobial agent in the medical field.

Toxicity to *Artemia salina*

Table 1 displays the results of the toxicity test involving the micro crustacean. The biosurfactant concentration of 600 mg/l demonstrated low toxicity to *A. salina* after 24 hrs of exposure (mortality index: 10 to 55%). The LC_{50} value was not identified at this concentration. The control samples (containing only seawater) did not cause the death of the micro crustacean. On the other hand, the LC_{50} values at biosurfactant concentrations of 200 and 400 mg/l were not calculated due to the low percentage of morality (less than 50%). Eighty percent of the microcrustaceans remained alive at a concentration of 200 mg/l and 70% remained alive at a concentration of 400 mg/l. These results demonstrate the low degree of toxicity of the biosurfactant tested.

The LC_{50} value was calculated using the trimmed Spearman-Karber method (Youn-Joo, 2006). The LC_{50} of the biosurfactant was 65.52% (v/v), ranging from 48.14 to 89.17% with a 95% confidence interval.

Table 1. Toxicity of biosurfactant isolated from *C. sphaerica* UCP 0995 cultured in distilled water supplemented with 5.0% soybean oil refinery residue and 2.5% corn steep liquor after 24 hrs of incubation using *A. salina* in samples of saline water as the bioindicator.

Biosurfactant solution	Mortality index (%) in saline water samples with extracts of isolated biosurfactant		
	25% extract	50% extract	75% extract
200 mg/l	20 ± 2.5	30 ± 3.0	40 ± 1.4
400 mg/l	30 ± 2.1	40 ± 2.2	60 ± 1.6
600 m/l	40 ± 1.7	60 ± 2.1	40 ± 3.1

Souza et al. (2005) investigated the toxicity of a biosurfactant produced by *P. aeruginosa* cultivated in glycerol in bioassays with *A. salina* and also found low degrees of toxicity at concentrations of 700 and 525 mg/l, with 100 and 50% lethality after 24 hrs, respectively, whereas no lethality was observed at lower concentrations of the biosurfactant or with the cell-free broth. Siqueira et al. (1998) demonstrated that the validity and reliability of toxicity bioassays using *A. salina* are related to the fractions that contain a recognized active substance. The LC_{50} value in the authors' experiments after 60 days of incubation was therefore not calculated using this method due to the low mortality index. The control (containing only seawater and *A. salina*) indicated that the larvae were not affected, thereby validating the conditions of the experiment, as all 10 larvae remained alive. According to Cavalcante et al. (2000), lethality assays allow the assessment of general toxicity and should therefore be considered essential to preliminary tests involving the study of compounds with potential biological activity. Using the *Scenedesmus subspicatus*, Reginatto et al. (1999) found that, although standardized, the toxicity tests involving this alga were not precise, as a more detailed international standardization is needed, taking into consideration the characteristics of the samples to be evaluated.

CONCLUDING REMARKS

The use of agroindustrial residues is an attractive alternative for the production of efficient biosurfactants, as the sources used in the present study are readily available and inexpensive. The isolated biosurfactant demonstrated no toxic effects against the microorganisms or micro crustacean studies or the germination of the seeds analyzed, thereby demonstrating its biotechnological potential in bioremediation processes in contaminated soil and water.

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