Research Paper

Characterization of new biosurfactant produced by *Trichosporon montevideense* CLOA 72 isolated from dairy industry effluents

Andrea S. Monteiro[1], Joana O. P. A. Coutinho[1], Ary C. Júnior[1], Carlos A. Rosa[1], Ezequias P. Siqueira[2] and Vera L. Santos[1]

[1] Departamento de Microbiologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil
[2] Laboratório de Química de Produtos Naturais, Centro de Pesquisas René Rachou Fundação Oswaldo Cruz, Belo Horizonte, MG, Brazil

The yeast strain CLOA 72 isolated from the effluent of a dairy industry in Brazil and identified as *Trichosporon montevideense*, was able to grow and produce a glycolipid biosurfactant when cultured on a mineral medium (MM) with sunflower oil as the carbon source. Biosurfactant production was partially growth-associated and maximal emulsification activity was observed at 144 h of cultivation (78.92%). The biosurfactant purified by precipitation with ethanol showed 78.66% emulsifying activity when used in concentrations above 4.5 mg/ml and was able to reduce the surface tension of water to values below 44.9 mN/m. The critical micellar concentration (CMC) was found to be 2.2 mg/ml. The highest emulsifying activity (E_{24}) has been observed with vegetable oils, toluene, kerosene, isooctane, cyclohexane, hexane, diesel oil and hexadecane as compared to mineral oil and oleic acid. The biosurfactant also showed good stability during exposure to 100 °C for different periods of time (10 to 60 min), to high salinity (30% of NaCl, KCl and $NaHCO_3$), and to a wide range of pH values (1–10). The biosurfactant purified by gel filtration chromatography is a glycolipid, with lipid portion containing 16.03% (9Z)-octadec-9-enoic acid, 14.92% hexadecanoic acid, and 9.63% (*E*) octadec-9-enoic acid and the carbohydrate portion containing mannose (35.29%), xylose (41.99%), arabinose (17.47%), and glucose (5.25%).

Keywords: *Trichosporon montevideense* / Biosurfactant / Emulsifying

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Introduction

Biosurfactants are amphiphilic compounds produced by a variety of microorganisms that contain hydrophobic and hydrophilic moieties. These compounds are able to accumulate between fluid phases, thus reducing surface and interfacial tension at the fluid surface and interface [1]. Biosurfactants have gained significance in the fields of enhanced oil recovery, environmental bioremediation, food processing, and pharmaceuticals. Interest in the production of biosurfactant has steadily increased during the past decade. The reasons for their popularity as high-value microbial products are based primarily on their specific action, low toxicity, higher biodegradability, effectiveness at extremes of temperature, pH and salinity, widespread applicability, and their unique structures which provide new properties when compared to synthetic surfactants [2, 3].

Unlike chemical surfactants, which are mostly petroleum-based, biosurfactants can be produced through microbial fermentation processes using a wide range of carbon sources, including cheap agricultural renewable resources like sugars, plant-derived oils, oil wastes, animal fat, starchy substances, molasses, lactic whey, and distillery wastes [4]. This fact reflects on the low costs of biosurfactant production and ensures benefits

Correspondence: Vera Lúcia dos Santos, Departamento de Microbiologia, ICB-UFMG, C.P. 486, 31270-901, Belo Horizonte, Brazil
E-mail: vlsantos@icb.ufmg.br
Fax: 55 (31) 499 2730

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over synthetic surfactants in terms of replacement of resources and recycling. However, large-scale production of these molecules has not been achieved because of the low yields of production and high recovery and purification costs [5, 6]. To circumvent these problems, it is imperative to isolate microorganisms capable of producing biosurfactants in an efficient manner and optimize massive production of an equally stable and highly active biosurfactant molecule from different substrates, including renewable resources and agro industrial residuals.

Even though biosurfactant production by microorganisms appears to be ubiquitous, environments contaminated with xenobiotic hydrophobic molecules are more prone to house good producers of novel biosurfactant molecules [7]. Traditional methods of screening from natural environments, particularly of unexplored or little explored niches, still continue to be a reliable way of isolating and increasing our inventory of new biosurfactants that may reveal potentially novel chemical and physical properties.

Yeasts are considered good producers of biotechnologically important molecules. However, few yeast strains are described in literature as producing biosurfactants, including *Yarrowia lipolytica*, *Kurtzmanomyces* sp., *Candida antarctica*, *Pseudozyma aphidis*, *C. glabrata*, and *C. utilis* [3, 8–12]. Among these, most are described to produce either a low-molecular-weight glycolipid biosurfactant or a high-molecular-weight polymeric molecule. Glycolipids most commonly produced are the sophorolipids and the mannosylerythritol lipids [13]. A polymeric biosurfactant is constituted of carbohydrates, proteins, and lipids [14].

Using an enrichment culture method we isolated a yeast strain (CLOA 72) from an aeration tank at an activated sludge wastewater treatment plant of a dairy industry, which is capable of producing a biosurfactant when cultivated in vegetable oil medium. Here we describe the physiological characteristics of the strain, its identification by molecular methods, the production of a biosurfactant molecule in a variety of substrates, and the primary characterization of the biosurfactant molecule.

Materials and methods

Isolation, selection and identification of biosurfactant producers

The CLOA 72 strain was isolated from an aeration tank in an activated sludge wastewater treatment plant of a dairy industry in Minas Gerais State, Brazil. Liquid enrichment was performed aerobically in mineral medium (MM), containing 3.4 g K_2HPO_4, 4.3 g KH_2PO_4, 0.3 g $MgCl_2 \cdot 2\ H_2O$, 1 g $(NH_4)_2SO_4$, 0.5 g yeast extract and 0.2 g chloramphenicol per liter. Flasks were supplemented with sunflower oil (20 g/l) as the sole carbon source and incubated at 25 °C on a reciprocal rotary shaker at 200 rpm for 96 h. Streak-plate isolation in potato dextrose agar medium (PDA; Biobras, Brazil), supplemented with chloramphenicol (0.2 g/l) was carried out and the plates were incubated at 25 °C for 120 h. Individual colonies on the plates were purified by streaking at least three times on PDA and evaluated by the emulsification index (E_{24}) method for biosurfactant production, as reported by Cameron *et al.* [15]. In these assays, 4 ml aliquots of the cell free filtrate, obtained from the culture cultivated for 144 h on sunflower oil (20 g/l) supplemented MM, were mixed with 6 ml of toluene in a test tube and vortexed vigorously for 2 min. After 24 h, the proportion of toluene emulsified was compared with the total volume of toluene added. The emulsification index (E_{24}) was estimated as the height of the emulsion layer divided by the total height and multiplied by 100.

The isolate with highest emulsifying activity and best growth rate in sunflower oil-supplemented MM was named CLOA 72 and selected for further characterization. The isolate was identified by the taxonomic keys of Kurtzman and Fell [16] and its identity was confirmed by comparing their D1/D2 variable domains of the large subunit rDNA sequence with other sequences retrieved from the GenBank. Genomic DNA was prepared from the yeast cultures after 48 h of incubation on YM agar using the methodology described by de Barros Lopes *et al.* [17]. The D1/D2 variable domains of the large rDNA subunit were amplified by PCR using primers NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) according to Lachance *et al.* [18]. The amplified DNA was concentrated, cleaned (Kit Wizard Plus SV Minipreps DNA Purification System; Promega, USA), and sequenced in a MegaBACE™ 1000 automated sequencing system (Amersham Biosciences, USA). The sequence was analyzed with the DNAMAN program, version 4.1 (Lynnon BioSoft, Vaudreuil, QC, Canada). For storage, the yeast was maintained in GYMP broth (w/v 2% glucose, 0.5% yeast extract, 1% malt extract, 0.2% NaH_2PO_4) at −80 °C.

Production and recovery of biosurfactant produced by strain CLOA 72

The inoculum was prepared by transferring cells grown on Sabouraud Agar (Difco, USA) for 48 h at 25 °C and washed in saline solution (NaCl 0.85% w/v) to MM.

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Readings of optical density (O.D.) at 600 nm were carried out and the absorbance values were used to calculate the inoculum's size to obtain flasks with a cell density of 0.02 O.D. (corresponding to 3.32 $\log_{10}$ cfu/ml). The growth and biosurfactant production assays were performed in 500 ml Erlenmeyer flasks containing 200 ml of MM supplemented with 20 g/l of sunflower oil and incubated in an orbital shaker (180 rpm) at 28 °C for 144 h. Growth, biosurfactant production, and emulsifying activity (E_{24}) were estimated after 6, 8, 12 h and 24/24h intervals until reaching 144 h of incubation. The cell numbers were monitored by yeast colony counts of appropriate decimal dilutions on Sabouraud agar. Later plates containing 30 to 300 yeast colonies after incubation at 25 °C for 48 h were used to calculate average number of colony forming units (cfu/ml).

For the recovery of biosurfactant, cells were removed from the culture broth by centrifugation at 5000 rpm for 20 min at 4 °C. The supernatant was filtered through a 0.45-μm Millipore membrane and four volumes of cold ethanol were added. The white precipitate formed was collected by centrifugation at 5000 rpm for 20 min and treated with chloroform–methanol (2:1, v/v), in order to remove residual oils, and dried at 60 °C to constant weight. The obtained values were used to estimate production (g/l) and volumetric production rate (g/l/h) for each time- point evaluated.

Cell surface hydrophobicity test

Microbial surface hydrophobicity was assessed by the microbial adhesion to the hydrocarbon method (MATH). Experiments were carried out using a procedure described by Rosemberg *et al.* [19] in which cells were grown on sunflower oil-supplemented MM for 144 h. After centrifugation at 5000 rpm for 10 min at 4 °C, cells were washed twice with PUM buffer (22.2 g $K_2HPO_4 \cdot 4\,H_2O$; 7.26 g KH_2PO_4; 1.8 g urea and 0.2 g $MgSO_4 \cdot 7\,H_2O$ in 1 l distilled water, pH 7.2) to an initial absorbance of 1.0 at 600 nm. Hexadecane (0.5 ml) and cell suspensions (2.0 ml) were vortexed in a test tube for 2 min and equilibrated for 10 min. The bottom aqueous phase was carefully removed with a Pasteur pipette and the O.D. at 600 nm measured. The adherence was expressed as the percentage decrease in optical absorbance of the lower aqueous phase following the mixing procedure, compared with that of the cell suspension prior mixing.

Effect of carbon source on CLOA 72 growth and biosurfactant production

Assays were performed in Erlenmeyer flasks containing 300 ml of MM supplemented with one of the following carbon sources (20 g/l): sunflower oil, glucose, sucrose or glycerol. A control flask without any supplementary carbon source was also prepared and assayed. The yeast cells previously grown in Sabouraud agar for 48 h at 25 °C were inoculated at initial cellular concentration of 0.02 O.D. The cultures were incubated in shaker with agitation of 180 rpm at 28 °C for 96 h. Samples were collected at 24 h intervals to determine cell growth by measuring O.D. at 600 nm and emulsifying activity.

Assays of emulsification

The effect of biosurfactant concentration on emulsifying activity (E_{24}) was evaluated using samples of the biosurfactant dissolved in deionized water in different concentrations (0.19 to 15 mg/ml). The ability of the biosurfactant (20 mg/ml) to emulsify some hydrophobic substrates was also studied by replacing the toluene in the emulsification assay by hexadecane, cyclohexane, hexane, octane, diesel, kerosene, mineral oil, oleic acid, cotton oil, sunflower oil, and toluene. All assays were performed in triplicate.

Surface tension and CMC determination

The surface tension of each sample was determined by the Ring method [20] using a KRUSS tensiometer (K10T Hamburgo, Germany) equipped with a 1.9 cm De Nouy platinum ring at room temperature (26 ± 1.5 °C). The measurements of surface tension in deionized water were used as controls. The critical micellar concentration (CMC) was determined by plotting surface tension as a function of biosurfactant concentration and considering the CMC as a point where the slope of the curve abruptly changes [7]. Concentrations ranging from 0.1 to 24 mg/ml of the biosurfactant were prepared in deionized water. All determinations were done in triplicate.

Stability studies

Stability studies were done using the purified biosurfactant at a concentration of 20 mg/ml. Samples of 4 ml were transferred to capped glass tubes containing 6 ml of sunflower oil as substrate. Emulsions were formed as described earlier. Sunflower oil was chosen for this test because it does not evaporate at 37 °C. After 24 h of rest, the emulsions were incubated at 4 °C or 37 °C for 30 d. Subsequently, the emulsifying activity was assessed by the index of emulsification method (E_{24}). The effect of NaCl, KCl, and $NaHCO_3$ (8% to 30%) electrolytes addition on emulsions stability was also determined in the same way after addition of the salt to the emulsions.

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To study the effect of pH on emulsifier activity, the biosurfactant (20 mg/ml) was dissolved in potassium chloride-HCl (200 mM, pH 1 and 2), sodium acetate (200 mM, pH 3, 4 and 5), acetic acid (200 mM, pH 4), sodium phosphate (100 mM, pH 6, 7 and 8), or glycine-NaOH buffer (200 mM, pH 9 and 10) and the emulsification activities were measured. The biosurfactant was also maintained at a temperature of 100 °C in water bath for 10, 20, 30, 40, 50, and 60 min, then cooled at room temperature and thereafter used for emulsification assay.

Purification and characterization of the biosurfactant

In the assays, biosurfactant was recovered from cell free broths of MM cultures supplemented with sunflower oil (20 g/ml) by precipitation with ethanol and by treatment with chloroform-methanol. The precipitate was dried at 60 °C to constant weight, dissolved in water deionizer (5 mg/ml) and filtered through a 0.45 μm Millipore membrane to exclude any insoluble matter. The extract obtained was characterized as presence of protein according to the Bradford method [21] using bovine serum albumin as a standard. Carbohydrates were determined by the phenol-sulphuric acid method using D-glucose as a standard [22]. Free lipid content was determined according to the method of Piretti *et al.* [23] with modifications. In the assays, samples of 500 mg were added into sealed flasks of 100 ml containing 10 ml 5 M NaOH in methanol (95%). After incubation at 100 °C for 5 h, the extract was neutralized with 1 ml HCl (37%) and incubated in rotatory shaker (200 rpm) at 25 °C for 10 min. An aliquot of 20 ml ethyl acetate was added and the flask was incubated under agitation for additional 15 min. The organic phase was recovered and evaporated at 50 °C for 24 h. The concentration of lipids in the resulting sample was expressed in milligrams of fatty acids per 100 mg of biosurfactant.

The crude water-soluble biosurfactant was then applied to a Sephacryl S-200 column (1.6 × 57 cm, Pharmacia K 16/70 column) coupled to a FPLC system (Pharmacia). The column was pre-equilibrated with deionized water and eluted with 0.1 M PBS buffer degassed. Fractions (2 ml) were collected with a flow rate maintained at 1.0 min/ml and monitored by absorbance at 280 nm record, total sugars (phenol-sulphuric acid method), total protein (Bradford method) and emulsification activity (E_{24}). The fractions with higher values of emulsifying activity and carbohydrates were pooled, lyophilized in a freeze dryer (Labconco. Corporation, Kansas City, MO), and characterized as to the composition of lipid and carbohydrate moieties.

In order to determine the fatty acid composition of lipid moiety of the biosurfactant, the extracted lipids according to the method of Piretti *et al.* [23] were methylated with boron fluoride-methanol reagent (14%) in proportions 1 ml reagent per 10 mg of lipid. The sample was added to 2 ml microtubes and incubated at 95 °C in a water bath for 15 min. Fatty acid methyl esters (FAMEs) were extracted three times with *n*-hexane and analyzed in a Varian model 3380 gas chromatograph using He as a carrier gas. A flame ionization detector (FID) and a CP-Sil 88 capillary column (50 m × 0.25 mm i.d.) were used. Column temperature was initially set at 170 °C for 1 min and programmed to rise linearly at 4 °C/min to 250 °C; the thermal program was halted isothermally at this temperature. Injector and detector block temperature was 250 °C. As a reference standard, a bacterial fatty acids–methyl ester mixture (TM 37, FAME, Mix 47885; Supelco, USA) was used. These procedures were carried out on crude and pure biosurfactants sample, without hydrolysis with 5 M NaOH, to verify the presence of free fatty acids.

The carbohydrate composition of biosurfactant was determined by gas chromatography and mass spectrometry. A lyophilized sample of biosurfactant (1 mg) was hydrolyzed in a sealed tube with 150 μl of 2 M trifluoroacetic acid (CF_3COOH) at 120 °C for 4 h. After evaporation the residue was washed twice with methanol and the sample was reduced with 1 M aqueous sodium borohydride ($NaBH_4$, 100 μl) and acetylated with a mixture of potassium acetate (100 μg) and acetic anhydride (100 μl) at 100 °C for 2 h. The excess reagent was removed by evaporation and then washed several times with ethanol. The alditol acetates were extracted with ethyl acetate and water (1:1, v:v) and analyzed by GC-MS (SHIMADZU, model QP 5050 A) equipped with column PTE-5-Supelco (30 m × 0.25 mm ID, 0.25 μm film) using He as a carrier gas. Column temperature was programmed from 100 °C (1 min) at 4 °C/min to 200 °C, followed by a gradient of 20 °C/min to 300 °C, and maintaining at this temperature for 5 min. These procedures were carried for crude and pure biosurfactant samples, without hydrolysis, to verify the presence of free carbohydrates in these samples. Sugar identification was made by comparing the relative retention times of sample peaks with standards. The sugar standards used for identification were purchased from Supelco USA: glucose, mannose, galactose, ramnose, fucose, ribose, arabinose, and xylose. The results were recorded and processed using Class 3.02 software (Shimadzu) and expressed in relative percentage of each sugar.

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Statistical analysis

The experimental data are presented in terms of arithmetic means of at least three replicates. All statistical calculations were made using Sigma-Stat 3.5 statistical software (Jandel Scientific, San Rafael, CA). Analyses of variance (ANOVA) and Duncan means comparison test with a significance level of 0.05 were applied. Pearson's correlation coefficient was used to test for a correlation between growth, E_{24} and biosurfactant production. Values of $P < 0.05$ were considered as statistically significant.

Results and discussion

Identification of biosurfactant-producing yeast

The yeast strain CLOA 72 was identified as *Trichosporon montevideense* based on its 26S rDNA sequence (GenBank accession number EU862820) and morphologic and biochemical properties. The presence of substances in the growth medium able to reduce surface tension up to a value of 45 mN/m was observed. This number is compatible with the range of described values for yeasts of the *Yarrowia* and *Candida* genus [8, 24]. Haba *et al.* [24] studying biosurfactant production by bacteria of genus *Pseudomonas* and yeasts, found that tenso-active substances produced by *C. glabrata* and *C. lipolytica* reduced surface tension to values ranging from 35 to 45 mN/m.

Growth kinetic and emulsifying activity of extracellular biosurfactant produced by *Trichosporon montevideense* strain CLOA 72

Growth of *T. montevideense* CLOA 72 and production of biosurfactant in MM supplemented with sunflower oil (20 g/l) is shown on Fig. 1. The emulsifier/biosurfactant production was detected during the exponential growth phase, however, when microbial growth ceases, biosurfactant production continues. About 70% of the produced biosurfactant was obtained during the growth exponential phase, in the first 80 h. These results indicate that production of biosurfactant is partially growth-associated, once the peak of biosurfactant production and biomass did not coincide. The maximum of biosurfactant production was delayed for about 40 h in relation to the total biomass production. Also, the volumetric production rates of the biosurfactant increased in the exponential phase (first 80 h of incubation) reaching values around 0.38 g/l/h. After this period a decline in production was observed (Fig. 1).

The highest emulsifying activity (E_{24}) of the cell-free culture broth samples was observed at 120 h of cultivation (78.92%), which is compatible with the maximum

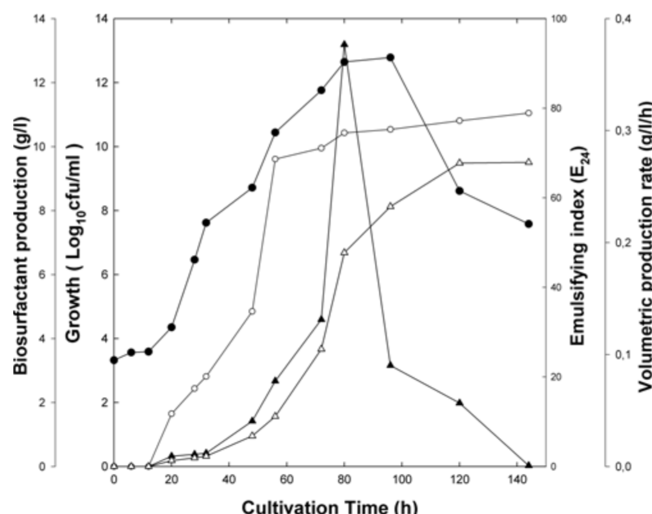

Figure 1. Time course of cell growth and biosurfactant production by *Trichosporon montevideense* CLOA 72 grown in a mineral medium with 2% sunflower oil and incubated in an orbital shaker at 180 rpm at 28 °C.
Log cfu/ml (–□–), Emulsifying index (E_{24}) of the culture medium (–○–), production of biosurfactant (g/l) (–△–) and volumetric production rate (g/l/h) (–▲–). The standard deviation from the means based on three replicate experiments was lower than 10%.

amount of biossurfactant produced. Emulsifying activity was linearly correlated with growth (linear correlation coefficient, $r = 0.977$, and Pearson correlation coefficient, $P = 0.0002$) and biosurfactant production ($r = 0.858$, $P = 0.0026$). In the first 56 h, the correlation between E_{24} and growth was even clearer ($r = 0.986$, $P = 0.0001$). Kinetics of partially or totally growth-associated biosurfactant production have also been reported for *Curvularia lunata* IM2901 [25], *Rhodococcus erytropolis* [26], *Pseudomonas aeruginosa* LBI [27] and *Bacillus subtilis* LB5a [6]. Biosurfactant production by microorganisms can be detected in different circumstances: (1) growth-associated (2) under growth-limiting conditions (3) by resting or immobilized cells; and (4) production with precursor supplementation. In the case of growth associated production, parallel relationships exist between growth, substrate utilization, and biosurfactant production [14]. In this study, it was observed a linear correlation between cellular concentration (cfu/ml) and biosurfactant production in the logarithmic phase ($r = 0.836$, and $P = 0.0026$). This behavior could be explained by the fact that the emulsifier was being produced to improve carbon source assimilation. Process biosurfactant-mediated oil assimilation can occur according to two ways: either making cellular surface more hydrophobic or the biosurfactant enhancing the aqueous solubilization and dispersion of the oils. During the growth of CLOA 72 in medium containing sun-

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flower oil there was no change in hydrophobicity of the cells (data not shown). This may suggest that the capture of sunflower oil involves emulsification and solubilization in the presence of biosurfactants.

Effect of various sources of carbon on growth and emulsifying activity of the strain CLOA 72

Cell density and emulsifying activity of *T. montevideense* CLOA 72 expressed by the index of emulsification (E_{24}) varied according to the source of carbon added to the growth medium (Fig. 2A and B). The highest values of cell density were observed with sunflower oil, followed by glucose, sucrose and glycerol. The emulsifying activity was detected after 48 h of growth for all substrates except for sunflower oil, in which the emulsifying activity was detected after 24 h. The carbon source used affected the emulsifying activity (Duncan test, $P < 0.05$) and the values of emulsifying activity for sunflower oil, glucose, sucrose and glycerol were 75.80, 32.40, 29.40, and 18.10%, respectively, after 96 h. These results are in agreement with those presented by several authors who have demonstrated the dependence of yield and structure of microbial surfactants on the carbon source and other available nutrients [14]. The metabolic pathways involved in the synthesis of the hydrocarbon and carbohydrate precursors of biosurfactants are diverse and, to some extent, dependent on the nature of the main carbon source [14]. Accordingly, the carbon substrate availability has been proposed by some researchers as either stimulator or repressor of the biosurfactant biosynthesis. Syldatk *et al.* [28] indicated the various possibilities that could occur for the biosurfactant's synthesis and assembly: (1) the hydrophilic and hydrophobic moieties are synthesized *de novo* by two independent pathways followed by their association to form a complete biosurfactant molecule, (2) the hydrophilic moiety is synthesized *de novo* while the hydrophobic moiety synthesis is substrate dependent, followed by their assembly, (3) the hydrophobic portion is synthesized *de novo* while the synthesis of hydrophilic portion is induced by substrate (4) both hydrophilic and hydrophobic moieties have substrate-dependent synthesis. Therefore, much attention has been focused on the regulation of physiological factors that could improve biosurfactant production efficiency.

The carbon sources generally used in biosurfactant production can be divided into three categories: carbohydrates, hydrocarbons, and vegetable oils. Some microorganisms produce biosurfactants by using only hydrophobic carbon sources, hydrocarbons, or vegetable oils; others use carbohydrates only; and some others may use several carbon sources either combined or individually [14]. Bonilla *et al.* [29] observed that *Pseudomonas putida* ML2 produces emulsifying activity when cultivated on a MM containing glucose, glycerol, ethanol, or naphthalene as the sole carbon source, but for glucose, the rate of emulsification is 10% greater than that observed when naphthalene is used as a sole carbon source. Because CLOA 72 grew and produced biosurfactants from all substrates tested, we believe that cheap agricultural renewable resource and wastes are promising carbon sources to be used as substrate for economic production of biosurfactant. For example, glycerol is a byproduct of the upcoming biodiesel industry, while sucrose can be obtained from the molasses and effluent of the sugar processing industry. We are now working on the optimization of culture media

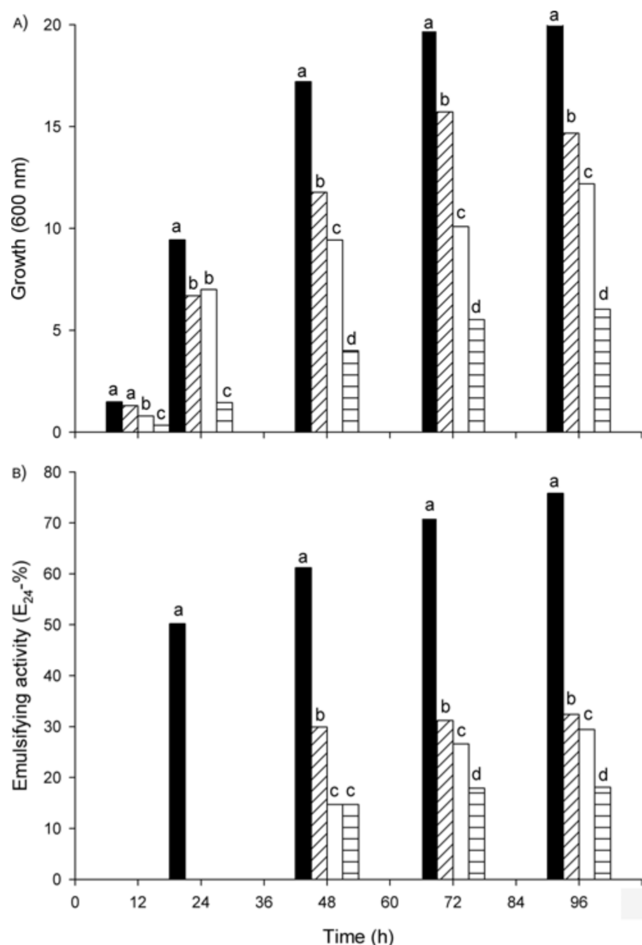

Figure 2. Growth (A) and emulsifying activity (B) of *Trichosporon montevideense* CLOA 72 in different carbon sources during 96 h in a mineral medium supplemented with one of the following carbon sources (g/l): sunflower oil (■), glucose (▨), sucrose (□) or glycerol (▤). The values standard deviation obtained from the dates of growth and E_{24} were less than 13 and 7% of the means, respectively. Different letters for each incubation time denote significant differences ($P < 0.05$) through the Duncan test.

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based in glycerol-containing biodiesel wastes to increase the productivity of the process and checking new applications for biosurfactant produced by CLOA 72.

Determination of the reduction of surface tension of water and critical micellar concentration

One of the important characteristics of a potent surfactant is its ability to lower the surface tension of aqueous solutions. The purified biosurfactant from *T. montevideense* CLOA 72 at various concentrations was dissolved in distilled water and its surface tension was measured. The CMC was 2.2 mg/ml and the surface tension as that point was 44.9 mN/m. The reduction levels of surface tension and the CMC of biosurfactants are dependent on the type of biosurfactant and its structure. For example, in general, glycolipids can reduce the surface tension of water up to 70% of its initial value, and can possibly reach values up to 25 mN/m [30]. The biosurfactant produced by CLOA 72 showed a smaller CMC value when compared to other biosurfactants from yeasts described in the literature, such as 10 mg/ml and 25 mg/ml for biosurfactants produced by *C. lipolytica* [31, 32], but higher than that produced by *Y. lipolytica* (0.5 mg/ml) [8].

Emulsifier activity

The relationship between biosurfactant concentration and emulsifying activity was tested using toluene as a hydrophobic substrate (Fig. 3). The emulsification index values went up with the increase in concentrations of the biosurfactant in aqueous phase until 4.5 mg/ml. The values ranged from 7.35% (assayed with 0.38 mg/ml of biosurfactant) to 78.66% (assayed with 4.5 mg/ml of biosurfactant). No significant emulsifying activity was observed when concentrations below 0.19 mg/ml were used. This is in agreement with results reported previously for a bioemulsifier produced by *Penicillium* sp., which presented saturation levels above 4 mg/ml [33].

In addition to surface tension, stabilization of an oil/water emulsion is commonly used as a surface activity indicator. Most microbial surfactants are substrate-specific and solubilize or emulsify different hydrocarbons at different rates. The emulsifier specificity (20 mg/ml) produced was assayed with various hydrophobic substrates (Fig. 4). The highest emulsifying activity values were obtained for vegetable oils, toluene, kerosene, isooctane, cyclohexane and hexane, which showed values near to 77.94%. Significant differences between the E_{24} indices were observed for the other compounds evaluated as follows: values were 73.55 for

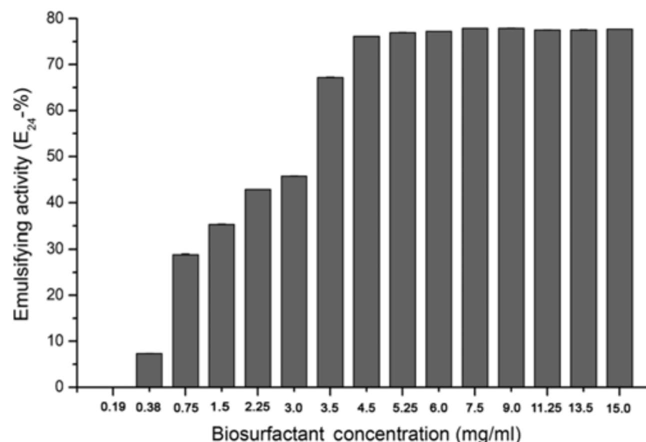

Figure 3. Effect of biosurfactant concentration on emulsifying activity expressed by emulsification index (E_{24}) using toluene as substrate.

diesel oil, 70.73% for hexadecane, 20.57% for mineral oil and 9.85% for oleic acid.

Several studies have shown that biosurfactants and bioemulsifiers may vary in their ability to emulsify different hydrophobic compounds [31]. It was suggested that the emulsifying activity depends on the affinity for hydrocarbon substrates, which involves a direct interaction with the hydrocarbon itself rather than an effect on the surface tension of the medium [31]. An emulsion is formed when one liquid phase is dispersed as microscopic droplets into another liquid continuous phase [14]. Poor emulsification of some hydrocarbons might be due to the inability of the biosurfactant to stabilize

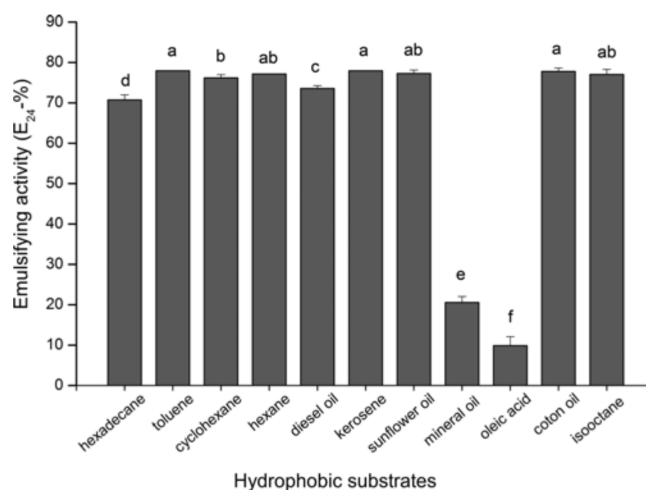

Figure 4. Emulsification of various hydrophobic substrates of the biosurfactant produced by *Trichosporon montevideense* CLOA 72 grown in a mineral medium supplemented with 20 g/l sunflower oil. Biosurfactant was dissolved to 20 mg/ml in sterile distilled water. Data are mean of three replicates, error bars represent standard deviation. Different letters denote significant differences ($P < 0.05$) through the Duncan test.

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the microscopic droplets. The emulsifying activity of broad spectrum is essential for biosurfactants to be used in industrial processes, such as treatment of industrial effluents, washing of deposits of containment of oily substances, and the pumping of heavy oils, considering that they have different mixtures of hydrophobic compounds to be emulsified. Biosurfactant produced by *T. montevideense* CLOA 72 seems to be a very effective and efficient emulsifier. Emulsan, one of the most effective emulsifiers, stabilizes Hydrocarbon-in-water emulsions (% hydrocarbon of 0.01–0.10) at low concentrations (0.02–0.2 mg/ml), but it exhibits considerable substrate specificity – it does not emulsify pure aliphatic, aromatic or cyclic hydrocarbons. However, all mixtures that contain an appropriate mixture of an aliphatic and an aromatic (or cyclic alkane) are emulsified efficiently [34].

Stability studies

The emulsifying activity of the biosurfactant was evaluated at pH values between 1 and 10. The activity remained stable at 78% in the evaluated pH range. Similar results were also obtained for other microorganisms. The activity of the *Penicillium* sp. bioemulsifier was stable over a wide range of pH (3 to 9) [33], and the emulsifier of *Y. lipolytica* was stable and active in pH values ranging from 2 to 10 [35].

The exposure of the biosurfactant at a temperature of 100 °C for up to 60 min did not affect the activity of the emulsifying agent, and the emulsification index values remained stable corresponding to 77.94%.

The addition of electrolytes sodium bicarbonate, sodium chloride, and potassium chloride was ineffective in promoting the coalescence of emulsions of toluene formed by the addition of the biosurfactant, even when the electrolytes were added in amounts above the limit of saturation (30%). No significant alteration to the height of emulsifier layer was observed during the incubation of emulsion in the presence of electrolytes. Most known biosurfactants are less stable over such salt concentrations [35, 36]. The largest stabilities were described for the biosurfactant produced by *Penicillium* sp., whose emulsifying activity was not impended by the addition of NaCl (5–20%) [33], and for the surface-active compound synthesized by *Bacillus subtilis* LB5, that retained its properties during exposure to 20% NaCl [6]. The electrolytes used in this study are widely used in industry for the treatment of liquid effluents or recovery of oil as biodiesel. Our results suggest that the biosurfactant shows potential for use in the formulation of emulsions that require the addition of salts of different ion characteristics.

Emulsions formed by the biosurfactant with sunflower oil remained stable on exposure to the temperatures of 4 °C and 37 °C for 30 d (Fig. 5). These emulsions have shown similar visual aspects, indicating that these can be stored at either 4 °C or 37 °C. Similar results have been reported previously for biosurfactants produced by *C. lunata* [25] and *P. aeruginosa* MT22 [24]. Emulsions should remain stable for a long period of storage, and it is not desirable that the characteristics of the product change by variations in temperature.

Chemical characterization of the biosurfactant

The chemical characterization of the crude biosurfactant showed a total content of carbohydrate of 50%, a content of lipid of 45%, and 0.92% of protein. A similar composition was reported for biosurfactants produced by other microorganisms including *Serratia marcescens* [38] and *Y. lipolytica* NCIM 3589 [35]. The fractionation profile of the crude biosurfactant, concerning total sugar and total protein content and emulsifying activity is presented in Fig. 6. The fractionation procedure allowed for the isolation of two distinct peaks, the first exhibited low content of protein and absence of emulsifying activity (from 26 to 32 min), and the second exhibited only contents of total sugar carbohydrate and emulsifying activity (from 36 to 58 min). These results suggest that crude biosurfactant was contaminated by non-covalently bound protein, which were removed by gel filtration. The second peak fractions were pooled, lyophilized and characterized as composition of lipid moiety and carbohydrate moiety.

Gas chromatograph analysis of the lipidic fraction of biosurfactant showed that (9Z)-octadec-9-enoic (C18:0), (E) octadec-9-enoic acid (C18:1n9t), and hexadecanoic acid (C16:0) are the major fatty acids accounting for 40.58% of the total fraction. Other fatty acids determin-

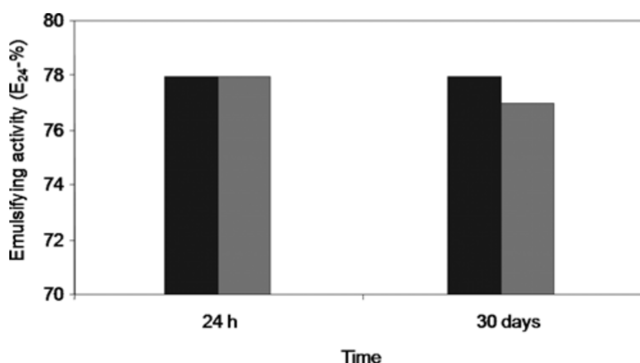

Figure 5. Stability of emulsifying activity of biourfactant produced by *Trichosporon montevideense* CLOA 72 stored at 4 °C (□) and 37 °C (■) for 30 d. Standard deviation values from the means based on three replicate experiments were lower than 0.1%.

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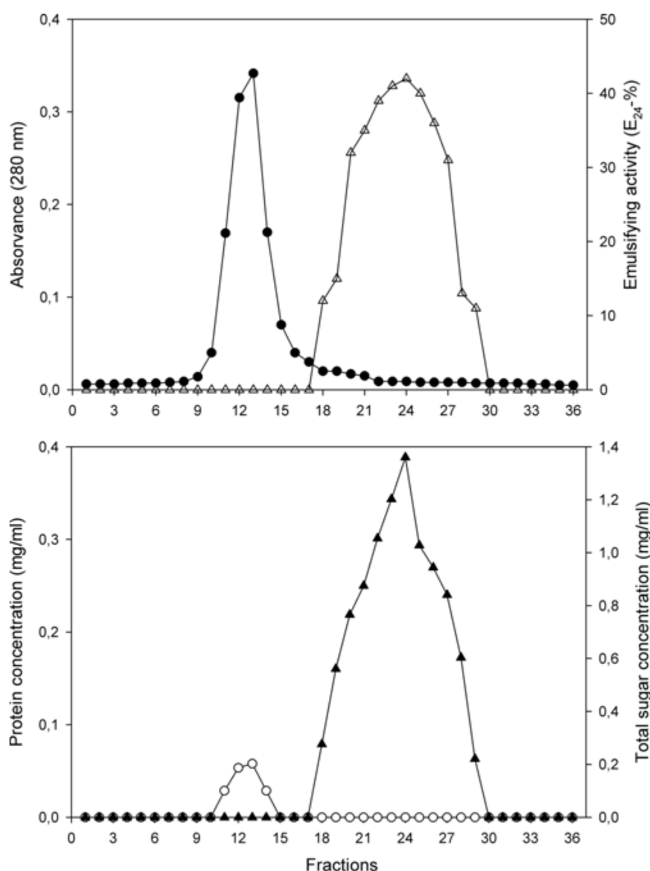

Figure 6. Elution profile of the crude biosurfactant obtained from *Trichosporon montevideense* CLOA 72 on Sephacryl S-200 column. Fractions were eluted with 0.1 M PBS buffer and monitored by absorbance at 280 nm record (–●–), total sugars (phenol-sulphuric acid method) (–▲–), total protein (Bradford method) (–○–) and emulsification activity (E_{24}) (–△–). Data are mean of three independent experiments.

ed at lower extent were (9Z)-octadec-9-enoic (C18:1n9c), 9-*cis*, *cis*, 12-octadecadienoic (C18:2n6c), *cis*-8, 11, 14, eicosatrienoic (C20:3n6), *cis*-15-tetracosenoic (C24:1n9). A similar composition of fatty acids has been described for surfactants produced by *Y. lipolytica* IMUFRJ 50682 [8] and *Penicillium* sp. [33]. The fatty acids were not detected from crude and pure biosurfactants samples, not hydrolyzed, suggesting their absence in these samples and the efficient removal with chloroform/methanol treatment.

The composition of carbohydrates in the biosurfactant was determined by CG-MS of alditol acetate derivatives. The monosaccharide composition was identified as xylose (41.99%), mannose (35.29%), arabinose (17.47%), and glucose (5.25%). In this analysis, sugars were not detected in the samples not hydrolyzed. These data suggest that free carbohydrates, not covalently linked to lipid, did not interfere with the chemical characterization of the molecule.

This composition is similar to that described for a surfactant produced by *Y. lipolytica* IMUFRJ50682 which shows a predominance of mannose [8], and a surfactant produced by *Y. lipolytica* NCIM 3589 composed of mannose and galactose [35]. In addition, a similar composition of sugars was observed for glycolipid produced by bacteria *Halomonas* sp. grown in hexadecane [37]. Biosurfactants of the hydrophilic group composed of a single type of monosaccharide has also been described, such as that produced by *C. lunata*, which presents only glucose in its constitution [33]. Microorganisms produce surface active substances classified into two main groups: (i) low molecular weight lipid-containing such as glycolipids, fatty acids, phospholipids and lipopeptides, and (ii) high molecular weight amphipathic molecules. The low-molecular-mass bioemulsifiers lower surface and interfacial tensions, whereas the higher-molecular-mass bioemulsifiers are more effective at stabilizing oil-in-water emulsions. The biopolymers usually consist of a combination of a hydrophilic polysaccharide backbone with additional hydrophobic components. The hydrophobic moiety necessary for emulsifications can be protein as in the emulsifier produced by *Acinetobacter calcoaceticus* BD4 or a lipid as in the RAG-1 emulsan of *A. calcoaceticus* RAG-1 [39, 34]. For the RAG-1 emulsan, the protein is not absolutely required for emulsifying activity, probably because of the hydrophobic fatty acids that contribute significantly to its activity [34]. The amphipathic compounds produced by *T. montevideense* CLOA 72 were able to reduce the surface tension and also make stable emulsions with different hydrophobic substrates.

Concluding remarks

The present study demonstrates that the biosurfactant produced from *T. montevideense* CLOA 72 presents emulsifying characteristics and is able to efficiently reduce surface tension. The biosurfactant stability over a wide range of temperatures, pH, and presence of electrolytes clearly demonstrates its potential for commercial applications that involve extreme environmental conditions. In particular, the strong emulsifying activity for the various tested hydrocarbons such as hexadecane, cyclohexane, hexane, octane, diesel, kerosene, mineral oil, oleic acid, cotton oil, and sunflower oil, as well as its stability at extreme temperatures confirm its suitability for the use in petroleum industry and environmental applications. The product's ability to form stable emulsions with vegetable oils suggests potential applications as cleaning and emulsifying agent in food industry. In addition, as CLOA 72 is able to grow and produced biosurfactants from sunflower oil, glycerol,

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glucose and sucrose, we believe that cheap agricultural renewable resource and wastes containing these substrates are promising carbon sources for economic production of this biosurfactant.

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