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New glycolipid biosurfactants produced by the yeast strain *Wickerhamomyces anomalus* CCMA 0358

Karla S. Teixeira Souza[a], Eduardo J. Gudiña[b,*], Zélia Azevedo[c], Victor de Freitas[c], Rosane F. Schwan[a], Lígia R. Rodrigues[b], Disney R. Dias[d], José A. Teixeira[b]

[a] Department of Biology, Federal University of Lavras (UFLA), Campus Universitário, 37.200-000, Lavras, MG, Brazil
[b] CEB – Centre of Biological Engineering, University of Minho, 4710-057 Braga, Portugal
[c] REQUIMTE – Laboratório Associado para a Química Verde, Departamento de Química e Bioquímica, Faculdade de Ciências, Universidade do Porto, Rua do Campo Alegre, 687, 4169-007 Porto, Portugal
[d] Department of Food Science, Federal University of Lavras (UFLA), Campus Universitário, 37.200-000, Lavras, MG, Brazil

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ABSTRACT

In this work, biosurfactant production by several yeast strains was evaluated using different culture media. The best results were obtained with the strain *Wickerhamomyces anomalus* CCMA 0358 growing in a culture medium containing glucose (1 g/L) and olive oil (20 g/L) as carbon sources. This strain produced 2.6 g of biosurfactant per liter after 24 h of growth. The crude biosurfactant reduced the surface tension of water to values around 31 mN/m, and its critical micelle concentration was 0.9 mg/mL. This biosurfactant was characterized through mass spectrometry (MS), and nuclear magnetic resonance (NMR) as a mixture of two different glycolipids, comprising a sugar moiety linked to one or three molecules of oleic acid. To the best of our knowledge, these biosurfactants are structurally different from those previously reported. Furthermore, the crude biosurfactant exhibited antimicrobial activity against several microorganisms, including the pathogens *Candida albicans*, *Escherichia coli*, *Staphylococcus epidermidis* and *Streptococcus agalactiae*, which opens the possibility for its use in several biomedical applications.

1. Introduction

Microbial surfactants or biosurfactants are a natural class of surface-active molecules with diverse structures that are produced by different microorganisms, including bacteria, yeasts and filamentous fungi. They are amphiphilic compounds, comprising a hydrophilic head and a hydrophobic tail, that are usually classified according to their chemical structure, being glycolipids and lipopeptides the most important classes [1,2]. Biosurfactants can be synthesized by microorganisms growing on water-immiscible substrates (e.g. vegetable oils, hydrocarbons) as well as on water-soluble compounds (e.g. carbohydrates, glycerol), and their production and composition are usually conditioned by the culture conditions and the composition of the culture medium [3–5]. These compounds play a critical role in the survival of their producing microorganisms, as they enhance the solubility of water-insoluble compounds (facilitating their transport into the cell), and participate in processes such as cell adhesion and aggregation, quorum sensing, biofilm formation and defense against other microorganisms [1,2].

Besides reducing surface and interfacial tensions in multi-phase systems, these molecules exhibit some interesting properties as compared to chemical surfactants, such as low toxicity and high biodegradability, improved environmental compatibility, ability to form foams and emulsions, as well as tolerance to extreme temperature, pH and salinity conditions [6–8]. For that reason, biosurfactants constitute an environmentally friendly alternative to traditional synthetic surfactants in several fields.

In addition, several biosurfactants exhibit antibacterial, antifungal and antiviral activities, which makes them relevant molecules for applications envisaging fighting diseases and infections [9–12]. Given the current efforts in searching and developing new agents that can replace traditional antibiotics, biosurfactants represent an extremely promising approach that ought to be further explored. Indeed, some biosurfactants have been described as viable alternatives to synthetic medicines and can be used as safe therapeutic agents, as anticoagulants and as anti-adhesive coatings on biomaterials [10,12–14]. Moreover, several biosurfactants exhibit

* Corresponding author at: CEB – Centre of Biological Engineering, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal.
E-mail address: egudina@deb.uminho.pt (E.J. Gudiña).

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anti-tumor activity and have shown potential to interfere with some cancer progression processes [1,15,16].

Most of the microbial surfactants reported are produced by bacteria such as *Bacillus* and *Pseudomonas* species [17,18]. However, due to the pathogenic nature of some of these microorganisms, the use of these compounds has been limited to few applications, often not being suitable for use in the food industry, among others. The evaluation of biosurfactant production by yeasts has increased in the last years, and several strains have been reported as promising biosurfactant producers due to their high production yields and their high substrate conversion rates; among them stand out species belonging to the genera *Candida*, *Starmerella*, *Pseudozyma* or *Yarrowia* [3,4,19,20]. Another great advantage of using yeasts for the production of biosurfactants is the GRAS (Generally Regarded as Safe) status that most of these strains exhibit, thus allowing the use of their products in the food and pharmaceutical industries. In addition, the microbial surfactants produced by yeasts are expected to present distinct structures and performances as compared to the commonly reported ones, which hold a great promise regarding the possibility of finding new bioactive agents.

The aim of this study was to evaluate the production of biosurfactants by five wild type yeast strains isolated from different environments, as well as to characterize potentially novel biosurfactants regarding their physicochemical and biological features.

2. Material and methods

2.1. Strains and culture conditions

The yeast strains used in this study were obtained from the Culture Collection of Agricultural Microbiology, CCMA (Department of Biology, Federal University of Lavras, Brazil), and included: *Yarrowia lipolytica* CCMA 0357 (isolated from Amazon soil); *Y. lipolytica* CCMA 0242 (isolated from Kefir water); *Wickerhamomyces anomalus* CCMA 0358 (isolated from coffee processing by-products); *Lindnera saturnus* CCMA 0243 and *Cryptococcus humicola* CCMA 0346 (both isolated from Cerrado soil). All the strains were grown in YEPG medium (10 g/L yeast extract; 20 g/L peptone; 20 g/L glucose, pH 6.5) at 28 °C. The following strains used in the antimicrobial assays were kindly provided by the Faculty of Pharmacy, University of Porto (Portugal): *Candida albicans*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus agalactiae*, *Streptococcus oralis* and *Streptococcus sanguis*. These strains were grown at 37 °C. *C. albicans*, *E. coli*, *P. aeruginosa*, *S. aureus* and *S. epidermidis* were grown in Luria-Bertani (LB) medium, whereas *S. agalactiae*, *S. oralis* and *S. sanguis* were grown in Todd-Hewitt Broth (THB). Both media were purchased from OXOID (Basingstoke, England).

2.2. Screening of biosurfactant-producing yeast strains

Eight culture media were used to evaluate the production of biosurfactants by the different yeast strains under study (Table 1). Olive oil and soybean oil were purchased from local markets. Olive oil mill wastewater (OMW), a residue generated during olive oil production, was obtained from an olive oil mill located in the north of Portugal. The assays were performed in flasks (500 mL capacity) containing 200 mL of the different media. Each flask was inoculated with a pre-culture of the corresponding yeast strain (grown overnight in YEPG medium at 28 °C and 200 rpm) to attain an initial cell concentration of 10^7 cells/mL. Subsequently, the flasks were incubated at 28 °C and 200 rpm up to 144 h. Samples (4 mL) were taken every 24 h to evaluate cell growth and biosurfactant production. The cell growth was determined according to the number of cells counted using a Neubauer improved cell counter (Marienfeld GmbH, Germany). Afterwards, the samples were centrifuged (2700*g*, 15 min) and the cell-free supernatants were used to assess the biosurfactant production through surface tension measurement and determination of the emulsifying activity, as described below.

2.3. Surface-activity measurement

The surface tension of the cell-free supernatants was measured according to the Ring method as described elsewhere [17]. A KRÜSS K6 Tensiometer (KRÜSS GmbH, Germany) equipped with a 1.9 cm Du Noüy platinum ring was used. All the measurements were performed in triplicate at room temperature (25 °C).

2.4. Emulsifying activity determination

The emulsifying activity was determined adding 2 mL of *n*-hexadecane to the same volume of cell-free supernatants in glass test tubes. The tubes were mixed with a vortex at high speed for 2 min and subsequently incubated at 25 °C for 24 h. The emulsification indexes (E_{24}, %) were calculated as the percentage of the height of the emulsified layer (mm) divided by the total height of the liquid column (mm) [7]. All the emulsification indexes were determined in triplicate.

2.5. Biosurfactant recovery

The yeast strain that offered the best results regarding biosurfactant production was selected to perform the following studies. The biosurfactant produced was recovered from the cell-free supernatant by adsorption chromatography, using a glass column (430 mL) filled with the polystyrene resin Amberlite XAD-2 (Sigma-Aldrich, USA), as described by Gudiña et al. [18]. Briefly, 250 mL of the cell-free supernatant obtained at the optimum fermentation time were passed through the column until the surface tension of the effluent was equal or higher than 50 mN/m. Subsequently, the column was washed with three volumes of demineralized water to remove the non-adsorbed compounds. Finally, the biosurfactant adsorbed to the resin was eluted with three volumes of methanol; the methanol was removed using a rotary evaporator at 40 °C and the product obtained was dissolved in a minimal amount of demineralized water and freeze-dried. The crude biosurfactant obtained was weighed and stored at −20 °C for further studies.

2.6. Quantification of residual oil

The amount of residual oil present in the culture medium along the fermentation was determined as described elsewhere [19]. Several 500 mL flasks containing 200 mL of culture medium were inoculated with a pre-culture of the corresponding yeast strain (as described above) and incubated at 28 °C and 200 rpm for different time intervals. A whole culture (200 mL) was used to recover the residual oil for each time point. Each sample was extracted three times with an equal volume of ethyl acetate; after the separation of the two layers, the ethyl acetate fraction (containing the residual oil) was recovered and vacuum-dried at 40 °C to remove the solvent. The residue obtained was washed twice with hexane to recover the oil present in the sample. After removing the hexane under vacuum at 40 °C, the amount of residual oil was determined gravimetrically. The assays were performed in triplicate for each time point, and the results were expressed as the average ± standard deviation.

Table 1
Composition (g/L) and surface tension values (ST, mN/m) of culture media evaluated for the production of biosurfactants by the yeast strains under study.

	Culture medium							
Composition (g/L)	I	II	III	IV	V	VI	VII	VIII
Glucose	5.0	25.0	25.0	25.0	1.0	1.0	1.0	1.0
Yeast extract	0.5	1.0	1.0	1.0	4.0	4.0	–	–
$(NH_4)_2SO_4$	0.5	–	–	–	–	–	4.0	4.0
NaCl	–	0.1	0.1	0.1	–	–	–	–
Glycerol	30.0	30.0	–	–	–	–	–	–
Olive oil	–	–	–	–	20.0	–	20.0	20.0
OMW	–	–	–	30.0	–	20.0	–	–
Soybean oil	–	–	30.0	–	–	–	–	–
Tween 80	–	–	–	–	–	4.0	4.0	–
KH_2PO_4	7.0	–	–	–	–	–	–	–
Na_2HPO_4	2.5	–	–	–	–	–	–	–
$MgSO_4{\cdot}7H_2O$	1.5	–	–	–	–	–	–	–
$CaCl_2{\cdot}2H_2O$	0.15	–	–	–	–	–	–	–
$ZnSO_4{\cdot}7H_2O$	0.02	–	–	–	–	–	–	–
$MnSO_4{\cdot}H_2O$	0.06	–	–	–	–	–	–	–
ST (mN/m)	53.4 ± 0.1	50.6 ± 0.1	48.6 ± 0.1	44.2 ± 0.1	52.4 ± 0.1	41.7 ± 0.1	37.5 ± 0.1	50.5 ± 0.1

2.7. Critical micelle concentration (cmc) calculation

Critical micelle concentration (*cmc*) is defined as the concentration of an amphiphilic compound in solution at which the formation of micelles is initiated. Different concentrations of the freeze-dried crude biosurfactant were prepared in demineralized water, and the surface tension of each sample was measured as described above. The *cmc* was determined by plotting the surface tension as a function of the logarithm of biosurfactant concentration, and it was found at the point of intersection between the two lines that best fit the pre- and post-*cmc* data, as described elsewhere [17]. All the measurements were performed in triplicate.

2.8. Biosurfactant chemical characterization

2.8.1. Mass spectrometry (MS)

A sample of crude biosurfactant was analyzed by direct injection of a methanolic solution into an Electrospray ionization source, ion-trap quadrupole mass spectrometer (LCQ DECA XP MAX, ThermoFinnigan) in positive and negative mode. Tandem mass spectra were obtained for the most intense ions to charge mass ratios.

2.8.2. Liquid chromatography–mass spectrometry (LC–MS)

A sample of crude biosurfactant was injected into a HPLC-DAD-UV (LCQ Thermo-Finnigan Surveyor) detection system using a previously reported method [21] with some modifications. The column used was a LiChroCART C18 column (4.6 × 150 mm; 5 μm), operating at 0.5 mL/min and 210 nm. The mobile phase was a gradient of acetonitrile and a solution of 1% of formic acid with an elution profile of 10% of acetonitrile for 5 min to 100% acetonitrile for an additional 10 min and 100% acetonitrile for more 30 min. The HPLC was connected to the mass spectrometer with an ESI probe at a spray voltage of 5 KV, capillary voltage of 4.5 V and capillary temperature of 325 °C, in positive and negative mode and data dependent analysis scan mode.

2.8.3. Transesterification/Silylation

An aliquot of 2 mg of crude biosurfactant sample was dissolved in methanol in the presence of catalytic amounts of sulfuric acid. The mixture was then extracted with *n*-hexane and the FAME (fatty acid methyl ester) recovered were analyzed by GC–MS. An aliquot of 2 mg of crude biosurfactant sample was mixed with hexamethyldisilazane (1 mL) in dimethylformamide (DMF) solvent (1 mL). The mixture was heated in oil bath at 100 °C during 1 h and the tetramethylsilane (TMS) fatty acids derivatives were analyzed by GC–MS. An aliquot of 1 mg of the crude biosurfactant sample was hydrolyzed with pectinase in sodium acetate buffer (pH 4.8) at 40 °C. The enzyme was precipitated with acetonitrile and the resulting solution, previously adjusted to pH 2 with glacial acetic acid, was evaporated to dryness in a rotary evaporator. The resulting fraction was silylated to the TMS derivatives and analyzed by GC–MS.

2.8.4. Gas chromatography–mass spectrometry (GC–MS)

FAME was analyzed by gas chromatography (GC) coupled to a single quadrupole mass detector. Separation was achieved using a TG-5MS capillary column (60 m long, 0.25 mm i.d., 1 μm film thickness). Mass spectrometry (MS) transfer line, ion source and injector temperatures were 300 °C, 280 °C and 300 °C, respectively. The mass detector was operating in the electron impact ionization (EIMS) with an electron energy of 70 eV. The initial column temperature was 150 °C, which was kept for 5 min, and then raised to 300 °C at a rate equal to 6 °C/min, where it was maintained for more 15 min. The injector was used in the splitless mode during 1 min. Helium was used as carrier gas at a constant flow rate of 1.2 mL/min. Mass ranges was from 40 to 1000 with scan times every 0.2 s.

2.8.5. Partial purification of the biosurfactant sample

The biosurfactant sample was purified by solvent extraction in two septs. Firstly, the crude biosurfactant sample was extracted with *n*-hexane and centrifuged. Secondly the resulting solid was extracted with ethyl acetate, and centrifuged again. The residue was kept for further analysis. The solvents were vacuum-dried removed and the two fractions, hexane fraction and ethyl acetate fraction, were analyzed by NMR and MS.

2.8.6. Nuclear magnetic resonance spectroscopy (NMR)

For the NMR analysis aliquots of each fraction were dissolved in CDCl3 in a 5 mm NMR tube.

^{1}H NMR (500.13 MHz) and ^{13}C NMR (125.77 MHz) spectra were recorded in CD_3OD/TFA (98:2) on a Bruker-Avance 500 spectrometer at 303 K and with TMS as an internal standard (chemical shifts (δ) in parts per million, coupling constants (J) in hertz). Multiplicities are recorded as singlets (s), doublets (d), triplets (t), doublets of doublets (dd), multiplets (m) and unresolved (*). ^{1}H chemical shifts were assigned using 2D NMR (COSY) experiment while ^{13}C resonances were assigned using 2D NMR techniques (*g*HMBC and *g*HSQC). The delay for the long range C/H coupling constant was optimized to 7 Hz.

2.9. Antimicrobial assays

The antimicrobial activity of the crude biosurfactant against several microorganisms was determined using the microdilution method in 96-well plastic tissue culture plates (Orange Scientific, Belgium), as described by Gudiña et al. [22]. Briefly, the crude freeze-dried biosurfactant was dissolved in demineralized water at a concentration of 5.2 g/L, and sterilized by filtration through a 0.2 μm pore-size filter. Subsequently, 125 μL of double strength culture medium (LB or THB, depending on the microorganism) were placed into the 1st column of the 96-well microplate, and 125 μL of single strength culture medium in the remaining wells. After that, 125 μL of the crude biosurfactant solution was added to the 1st column of the microplate and gently mixed with the medium. Subsequently, 125 μL from the 1st column was transferred to the 2nd column and mixed; serially, 125 μL were transferred to the subsequent wells, discarding 125 μL of the mixture in the 10th column, so that the final volume in each well was 125 μL. This process resulted in two-fold serial dilutions of the biosurfactant in the first 10 columns. 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 a pre-culture of the corresponding microorganism grown overnight in the appropriate culture medium (LB or THB) at 37 °C. The microplates were covered and incubated for 24 or 48 h (depending on the microorganism) at 37 °C. After the incubation time, the optical density at 600 nm was determined for each well. The growth inhibition percentages at different biosurfactant concentrations for each microorganism were calculated as:

$$\%\text{growthinhibition}_c = [1 - (OD_c/OD_0)] \text{ x } 100, \quad (1)$$

where OD_c represents the optical density of the well with a biosurfactant concentration c, and OD_0 is the optical density of the control well (without biosurfactant). Triplicate assays were performed for each microorganism and biosurfactant concentration.

3. Results and discussion

3.1. Screening of biosurfactant-producing yeast strains

The yeast strains under study were grown in different culture media (Table 1) in order to evaluate their potential as biosurfactant producers. Since biosurfactants reduce the surface tension of the medium, this potential was assessed as the difference between the surface tension at time zero (i.e. no biosurfactant produced) and the lowest surface tension value obtained along the fermentation (Table 2). The yeast strains were considered biosurfactant producers when this difference was equal or higher than 8 mN/m, as reported elsewhere [22].

The carbon sources used to evaluate biosurfactant production included glycerol (media I and II), olive oil (media V, VII and VIII) and soybean oil (medium III), which have been used previously as substrates for biosurfactant production by yeasts [4,19,21,23,24]. The agro-industrial waste OMW was also evaluated as a potential low-cost carbon source (media IV and VI). The culture media were formulated according to previous reports [4]. In all the cases, the different lipophilic substrates (olive oil, OMW and soybean oil) were used in combination with glucose, as it has been reported that biosurfactant production by yeasts is higher when hydrophobic and a hydrophilic carbon sources are supplied together in the culture medium [5,19,20,23,24].

Biosurfactant production was observed with three of the culture media assayed. Using medium I, which contains glycerol and glucose as carbon sources, *Y. lipolytica* CCMA 0357 and *W. anomalus* CCMA 0358 were identified as biosurfactant producers. In both cases, the surface tension of the culture medium was reduced to values around 43 mN/m, being this reduction achieved earlier (72 h) in the case of *W. anomalus* CCMA 0358 (Table 2). Glycerol is a readily available substrate, and large amounts of raw glycerol are expected to be available as by-products of biodiesel industries at low cost, making it an inexpensive carbon source for the production of different metabolites, including biosurfactants. Several studies reported the ability of *Y. lipolytica* of producing added-value compounds (including biosurfactants) using waste glycerol as carbon source [4,25]; however, higher surface tension reductions than the obtained in this work (up to 22 mN/m) have been reported for other *Y. lipolytica* strains using similar culture media [4].

Biosurfactant production was also observed using the media V and VIII (both of them containing olive oil). With these media, the surface tension reductions achieved with all the yeast strains studied were enough to consider them biosurfactant producers. The best results were obtained with *W. anomalus* CCMA 0358 growing in medium V, which reduced the surface tension to values around 36 mN/m after 24 h, followed by the *Y. lipolytica* strains (Table 2). Olive oil and oleic acid (the main fatty acid present in olive oil) have been successfully used, in combination with glucose, for the production of biosurfactants by different yeasts, including *Starmerella bombicola* and species belonging to the genera *Candida* and *Wickerhamiella*, among others [8,19,23,24].

OMW, an agro-industrial waste generated during olive oil production, was also used as a substrate to evaluate biosurfactant production by these yeasts. Although biosurfactant production by yeasts using OMW has not been previously reported, it was selected as it contains long-chain fatty acids similar to those present in olive oil (including palmitic, stearic, oleic and linoleic acid) [26]; furthermore, OMW has been successfully used as an inducer of biosurfactant production by *Pseudomonas aeruginosa* [26]. OMW is a significant pollutant that represents a waste disposal issue in countries that produce large amounts of olive oil, such as Portugal. Consequently, its use as substrate to produce biosurfactants is interesting from an economical and environmental point of view. However, using the culture media containing OMW (IV and VI), none of the yeast strains under study was able to produce biosurfactants. Soybean oil has also been used as carbon source to produce biosurfactants by different yeasts [19,21]. However, in this case, none of the isolates exhibited biosurfactant production using the culture medium containing soybean oil (medium III).

Besides the ability of reducing the surface tension, also the emulsifying activity was evaluated for all the yeast strains grown in the different culture media; however none of them was found to produce surface active compounds able to form stable emulsions under the studied conditions. Other authors reported previously that some biosurfactants produced by yeasts are not particularly effective emulsifiers, which excludes them from a number of applications, but makes them suitable for non-foam requiring purposes [2,6]. However, in other cases, good emulsifying activity with different hydrophobic substrates has been reported for biosurfactants produced by yeasts [8,19,20].

Y. lipolytica and *C. humicola* strains have been previously reported as biosurfactant producers [4,27]. Regarding *W. anomalus* (formerly known as *Pichia anomala*), only the strain PY1 has been reported to produce biosurfactants [21]. However, to the best of our knowledge, this is the first report of biosurfactant production by a *L. saturnus* strain.

Taking into account the surface tension reductions obtained, *W. anomalus* CCMA 0358 growing in medium V was selected to further characterize its growth and biosurfactant production kinetics (Fig. 1). This yeast strain achieved the lowest surface tension value (36.4 ± 0.7 mN/m) after 24 h of fermentation; at this point, the number of cells was 2.4×10^8 cells/mL. After that, an increase in the surface tension values was observed, which can be due to

Table 2
Surface tension reductions obtained at the optimum time (h) with the yeast strains grown in different culture media. Culture media composition is given in Table 1. Only those media where surface tension reductions were observed are presented. Results represent the average of triplicate experiments ± standard deviation.

Yeasts	Culture medium					
	I	II	III	V	VI	VIII
Yarrowia lipolytica CCMA 0357	10.8 ± 0.4 (120)	6.0 ± 0.1 (96)	1.5 ± 0.3 (120)	12.4 ± 0.3 (24)	4.1 ± 0.8 (120)	10.5 ± 0.1 (72)
Yarrowia lipolytica CCMA 0242	1.9 ± 0.2 (24)	2.6 ± 0.8 (24)	1.0 ± 0.7 (72)	12.3 ± 0.1 (96)	2.1 ± 0.3 (24)	10.3 ± 0.1 (72)
Cryptococcus humicola CCMA 0346	4.1 ± 0.6 (120)	4.4 ± 1.4 (96)	5.6 ± 1.1 (72)	8.9 ± 1.1 (24)	1.3 ±0.9 (24)	8.0 ± 0.3 (48)
Lindnera saturnus CCMA 0243	0.4 ± 0.1 (96)	4.6 ± 1.3 (96)	0.7 ± 0.4 (120)	9.5 ± 0.1 (72)	0.7± 0.0 (24)	9.9 ± 0.1 (24)
Wickeramomyces anomalus CCMA 0358	10.9 ± 1.0 (72)	5.2 ± 0.1 (24)	3.5 ± 0.4 (24)	16.0 ± 0.1 (24)	1.7 ± 0.1 (72)	10.1 ± 0.7 (24)

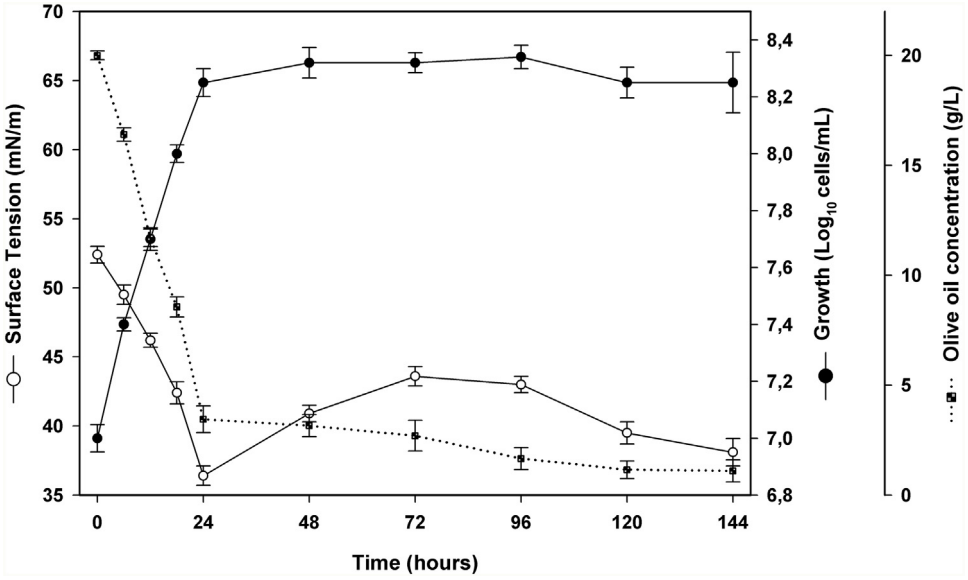

Fig. 1. Growth, biosurfactant production and olive oil consumption kinetics of *Wickerhamomyces anomalus* CCMA 0358 in culture medium V. The composition of this culture medium is shown in Table 1. Results represent the average of three independent experiments ± standard deviation.

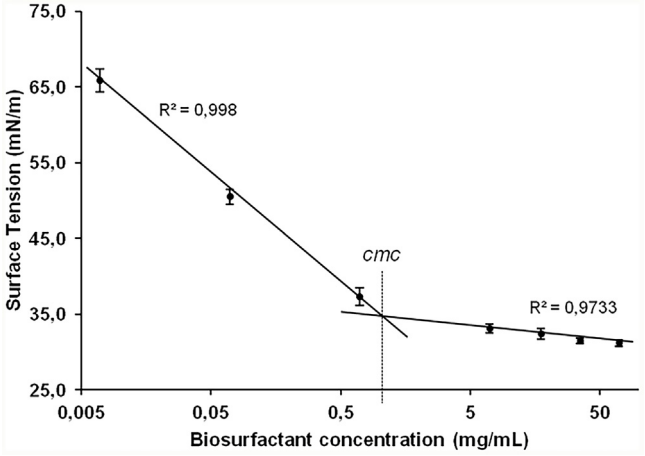

Fig. 2. Surface tension values (mN/m) *versus* logarithm of biosurfactant concentration (mg/mL) obtained with the crude biosurfactant produced by *Wickerhamomyces anomalus* CCMA 0358 dissolved in demineralized water. Results represent the average of three independent measurements ± standard deviation.

the partial degradation of the biosurfactant previously produced, whereas the number of cells remained almost constant up to 96 h (Fig. 1). These results suggest that biosurfactant production by *W. anomalus* CCMA 0358 in medium V is growth-associated. Similar biosurfactant production profiles were also reported by other authors for several yeast strains [19,21]. More than 80% of the olive oil originally present in the culture medium V was consumed by *W. anomalus* CCMA 0358 during the first 24 h of growth, corresponding to the highest biosurfactant production. At the end of the fermentation (144 h) the amount of residual olive oil present in the culture medium was reduced up to 1.1 ± 0.5 g/L (Fig. 1). Although the use of olive oil for biosurfactant production can be disadvantageous from an economical point of view, it has to be pointed out that, in the case of other yeast strains, the optimum concentration of hydrophobic carbon sources (including olive oil and oleic acid) used for biosurfactant production is considerably higher (between 40 and 100 g/L) [5,10,19–21,23,24].

3.2. Critical micelle concentration (cmc)

The *cmc* is the minimum concentration of a surface-active compound necessary to reduce the surface tension to the maximum extent; once achieved the *cmc*, further increases in its concentration do not have effect in the surface tension, which remains stable. The *cmc* is a property characteristic of each surface-active compound, and it is commonly used to define its efficiency. The lowest surface tension value that can be achieved with a surface-active compound is also a characteristic property, and it is used to define its effectiveness. The *cmc* calculated for the crude biosurfactant produced

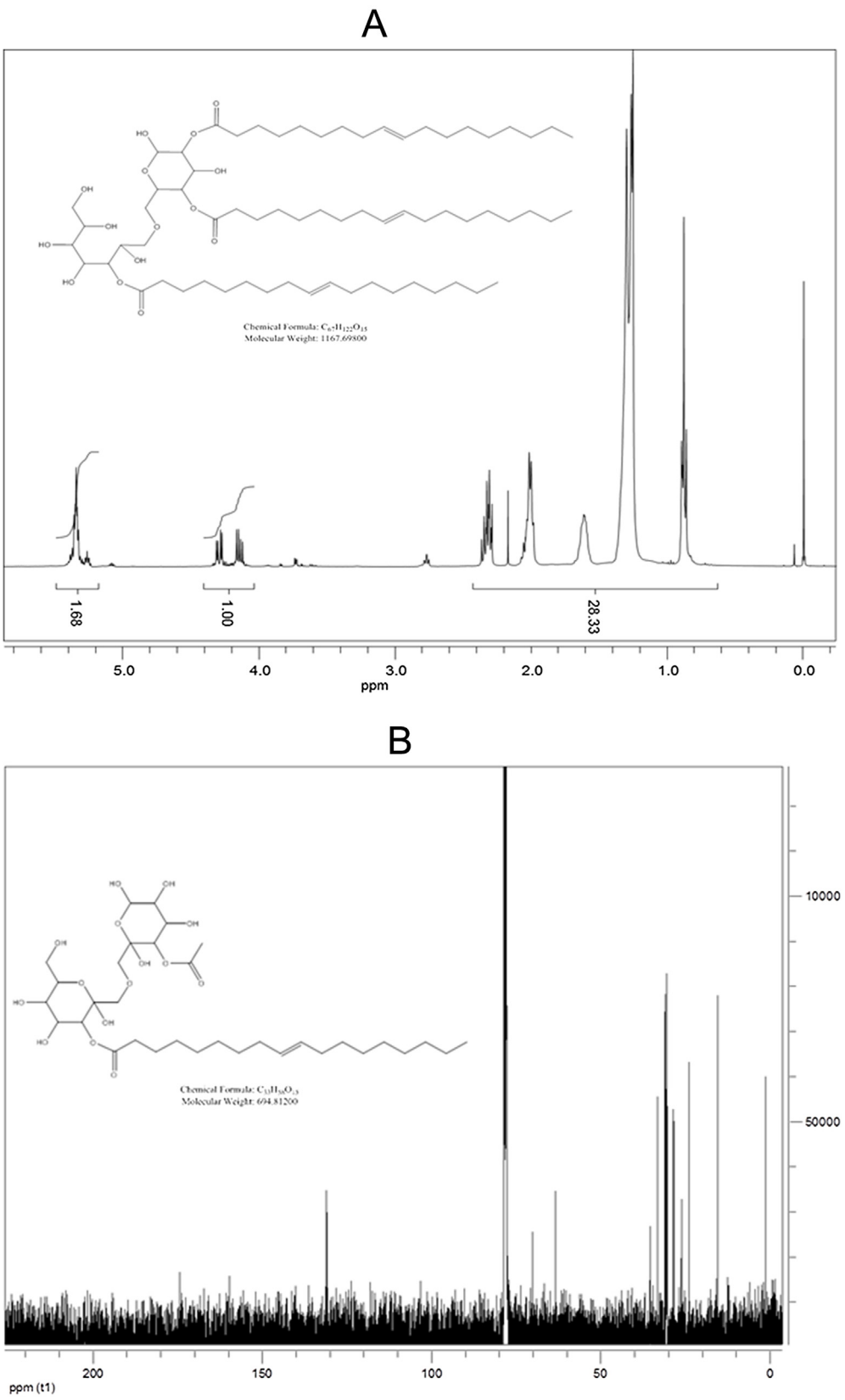

Fig. 3. ^{1}H (A) and ^{13}C (B) NMR spectra obtained from the ethyl acetate fraction of the biosurfactant produced by *Wickerhamomyces anomalus* CCMA 0358.

by *W. anomalus* CCMA 0358 in medium V was 0.9 mg/mL, and the minimum surface tension value was 31.2 ± 0.4 mN/m (Fig. 2).

The lowest surface tension value obtained for the crude biosurfactant produced by *W. anomalus* CCMA 0358 was similar to those reported for other biosurfactants produced by yeasts (between 30.3

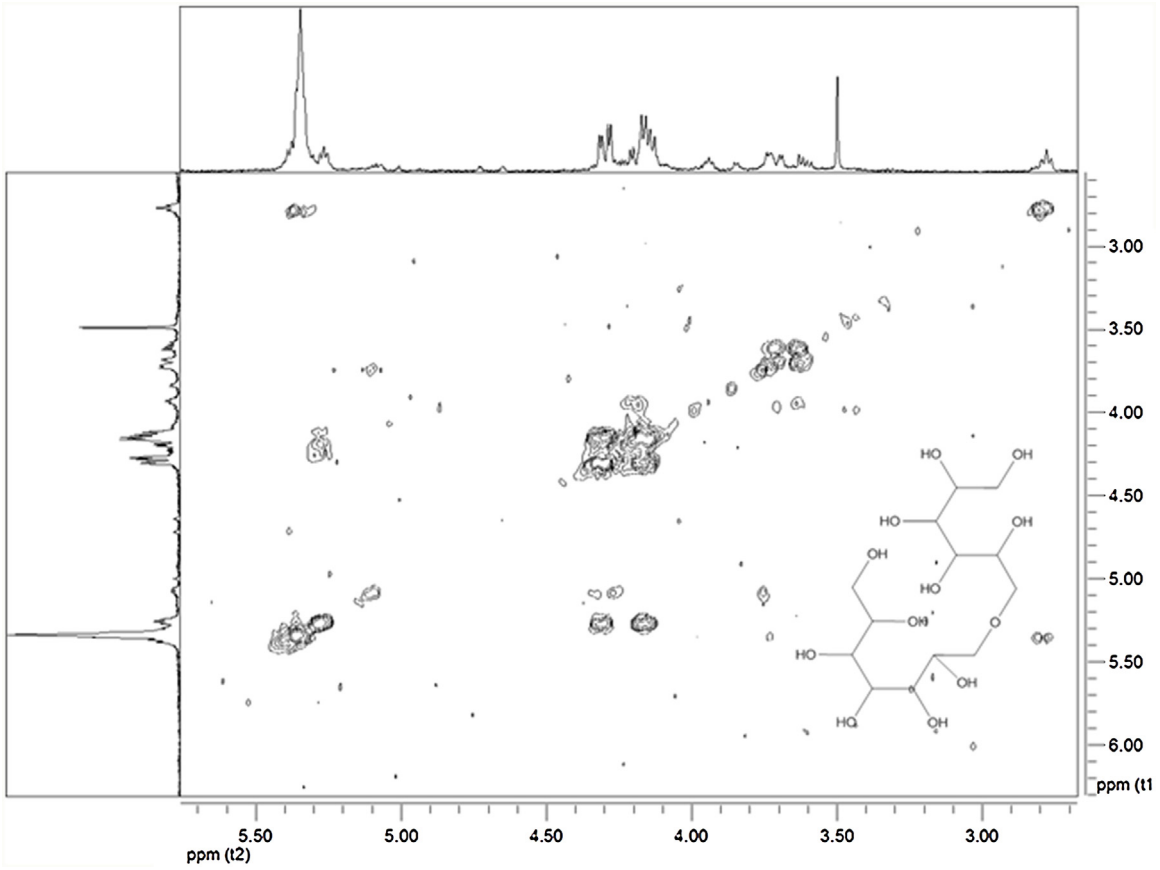

Fig. 4. Cosy NMR spectrum (amplified in the range of 3–6 ppm) obtained from the ethyl acetate fraction of the biosurfactant produced by *Wickerhamomyces anomalus* CCMA 0358.

and 35.9 mN/m) [5,6,8,19,20,27]. However, the *cmc* herein obtained was higher when compared with some values reported by other authors (0.02-0.366 mg/mL) [6,8,10,19,23], which may be due to differences in the purification processes.

Nevertheless, it is important to notice that the productivity reported in the current study is higher when compared with other examples from the literature. Different authors reported productivities between 1.2 and 62.5 mg/L/h for *Candida sphaerica*, *P. anomala* or *S. bombicola* strains [10,20,21]. In the present work, 2.6 g/L of biosurfactant was produced by *W. anomalus* CCMA 0358 after 24 h of growth, which results in a productivity of 108.3 mg/L/h.

3.3. Biosurfactant chemical characterization

3.3.1. GC–MS analysis

The partially purified biosurfactant produced by *W. anomalus* CCMA 0358 in medium V after 24 h of growth was transesterified/silylated to the corresponding FAME or TMS fatty acids in order to determine its fatty acid composition. Oleic acid appears to be the principal fatty acid present in the biosurfactant sample. The identification was based in other FAME samples analysis and on the MS NIST Library. Moreover, it was not observed any peak of methylated or silylated sugar or other significant compound on the GC–MS chromatogram obtained (Fig. A1). The silylated hydrolyzed sample showed the presence of two major TMS sugar by its mass spectra; however it was not possible to clearly identify its structure (data not shown).

3.3.2. NMR analysis

To further understand the biosurfactant molecule structure, the sample was purified by extractions with increasing polarity of organic solvents, hexane and ethyl acetate, leading to two main fractions. These fractions were analyzed by ^{1}H and ^{13}C NMR (Fig. 3A and B, respectively). The NMR spectrum of each fraction confirmed the presence of a fatty acid, similar to the oleic acid ^{1}H and ^{13}C NMR. Cosy NMR spectrum indicated that this fatty acid moiety is linked to a C—O symmetric template (Fig. 4). ^{13}C NMR spectrum showed the presence of a double bond with signals at 130 ppm. In addition, several CH_2 groups resonated at 22.7–34 ppm and a signal at 14.1, corresponding to a primary methyl group, confirmed that the fatty acid could not be a hydroxyl fatty acid. The spectrum also revealed signals of C—O bond at 62 and 69 ppm which could be from glycerol chain or C6 sugar carbon. No signals were resonated near 112 ppm (C1 sugar carbon). ^{13}C NMR spectrum of hexane fraction reveals a peak at 173.4 ppm and the one of ethyl acetate fraction shows an additional peak at 158 ppm, possibly due to the carbonyl of the acetyl group. However, the NMR spectrum of the hexane fraction was very similar to the one of a triglyceride sample [28].

3.3.3. MS analysis

ESI–MS analysis by direct infusion of the biosurfactant sample dissolved in methanol, in positive mode, showed a major peak at $m/z = 903$. Tandem MS/MS of this pseudomolecular ion revealed a minor and a more abundant ion with masses of 885 and 603, respectively. No ions were detected in the negative mode analysis. This behavior is very similar to the one of triglycerides. To elucidate the composition of the biosurfactant sample, it was further analyzed by LC–MS, with UV detection at 210 nm, in positive and negative mode. Ion extracted chromatogram of masses $m/z = 608/652/696$ and $m/z = 904/948/992$ in positive mode showed two peaks at 15 and 26 min (Fig. A2) corresponding to the very small peaks in the UV chromatogram (*data not shown*).

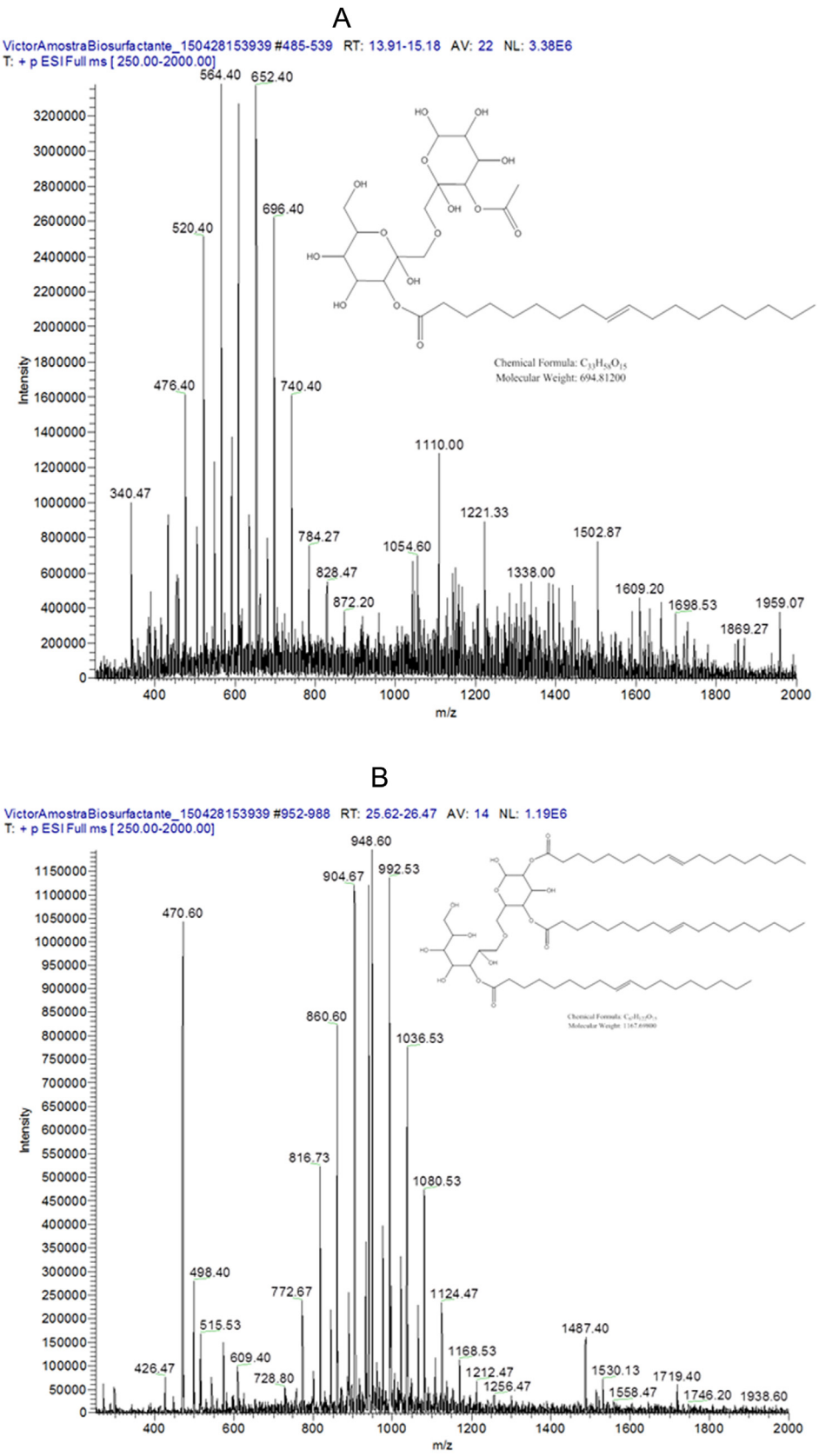

Fig. 5. Mass spectra of the glycolipids identified in the peaks at 15 min (A) and 26 min (B).

Table 3
Growth inhibition percentages obtained for different microorganisms exposed to the crude biosurfactant (2.6 g/L) produced by *Wickerhamomyces anomalus* CCMA 0358. Results are expressed as means ± standard deviations of values obtained from triplicate experiments.

Microorganism	% Growth Inhibition
Candida albicans[a]	78 ± 1
Escherichia coli[a]	16 ± 4
Pseudomonas aeruginosa[a]	–
Staphylococcus aureus[a]	–
Staphylococcus epidermidis[a]	66 ± 5
Streptococcus agalactiae[a]	100 ± 1
Streptococcus oralis	100 ± 2
Streptococcus sanguis	95 ± 5

[a] Pathogenic microorganisms.

The mass spectra of these peaks is presented in Fig. 5A and B, which indicates the presence of multiple mass peaks with a Gaussian distribution that differs from each other by differences of $m/z = 18$ or $m/z = 44$. This mass spectra behavior has already been reported by Daverey and Pakshirajam [29].

According to the ESI–MS direct infusion, no important peaks were detected in LC–MS analysis in negative mode, which means that no important free acidic group is present in the biosurfactant molecule. Tandem MS fragmentation of each mass peak present in the mass spectrum showed a very similar fragmentation pattern, with the presence of new fragment masses by the addition of 18 Da (MS^2 data dependent analysis) and 44 Da (MS^3data dependent analysis).

The LC–MS analysis of the hexane fraction revealed the presence of a peak that elutes at 26 min, with a mass spectrum similar to the one shown in Fig. 3B, but it was contaminated with some triglycerides. So ethyl acetate fraction was used for further NMR characterization.

Therefore, the proposed structure of the biosurfactant is presented in Fig. 5A and B, with a mass to charge ratio (m/z) of 696 (at 14.7 min) and 1168 (at 26 min).

According to the results obtained, the biosurfactant produced by *W. anomalus* CCMA 0358 is a glycolipid, being the lipophilic fraction composed by oleic acid. Two different types of glycolipid biosurfactant structures were detected, containing one (Fig. 5A) or three (Fig. 5B) molecules of oleic acid. Additionally, although the structure of the sugar moiety could not be completely resolved, it was concluded that it was not sophorose neither trehalose, as reported for other glycolipid biosurfactants produced by different yeasts [6,8,19,23].

P. anomala PY1 was previously reported to produce different sophorolipids in culture media containing glucose or soybean oil as carbon sources, with molecular weights of 675/691/707 Da and 658/675/691 Da, respectively [21]. The glycolipid biosurfactants produced by *W. anomalus* CCMA 0358 are different from those produced by *P. anomala* PY1, as they are not sophorolipids and their molecular weights are 696 and 1168 Da.

To the best of our knowledge, the chemical structure and the molecular weight of the biosurfactants produced by *W. anomalus* CCMA 0358 have not been previously described in the literature, and therefore represent new surface-active agents.

3.4. Antimicrobial activity

Microbial surfactants can interfere in the adhesion of microorganisms to different surfaces [11,13,14,30,31]; some of them have been reported to exhibit antibacterial and antifungal activity [9,22,32,33], and in some cases anti-tumor activity, interfering with some cancer progression processes [1,16]. These properties contribute to their potential use as alternatives to conventional therapeutic agents in many biomedical applications.

The antimicrobial activity of the crude biosurfactant produced by *W. anomalus* CCMA 0358 was determined by measuring the growth inhibition percentages obtained for different microorganisms at different biosurfactant concentrations. The results obtained at the highest biosurfactant concentration tested (2.6 g/L) are shown in Table 3. This biosurfactant exhibited high growth inhibition percentages (95–100%) against *S. oralis*, *S. sanguis* and *S. agalactiae*. A considerable inhibitory activity was also observed against *C. albicans* (78%) and *S. epidermidis* (66%). The lowest antimicrobial activity was observed against *E. coli* (16%), whereas no inhibitory activity was observed against *S. aureus* and *P. aeruginosa* (Table 3).

Similarly to the results herein obtained, the biosurfactant produced by *Candida lipolytica* UCP 0988 also exhibited lower antimicrobial activity against *P. aeruginosa*, *S. aureus* and *E. coli* when compared with *S. oralis*, *S. sanguis* and *S. agalactiae* [12]. Furthermore, the crude biosurfactant produced by *W. anomalus* CCMA 0358 exhibited higher antimicrobial activities against all the microorganisms tested (with the exception of *S. aureus* (27.3%) and *P. aeruginosa* (12.5%)) when compared with the biosurfactant produced by *C. sphaerica* UCP 0995 at a similar concentration (2.5 g/L), although the *cmc* of this biosurfactant (0.25 mg/mL) was considerably lower when compared with the one produced by *W. anomalus* CCMA 0358 [10].

Also biosurfactants produced by the lactic acid bacteria *Lactococcus lactis* 53, *Streptococcus thermophilus* A, *Lactobacillus paracasei* sbsp. *paracasei* A20 or *Lactobacillus agilis* CCUG31450 exhibited antimicrobial activity against the same bacterial strains herein studied, although at higher concentrations (3–100 g/L), and in most of the cases, the antimicrobial activities observed were lower when compared with the ones herein reported [9,13,22].

4. Conclusions

The yeast strain *W. anomalus* CCMA 0358 produced a mixture of glycolipid biosurfactants in a culture medium containing olive oil and glucose as carbon sources. The crude biosurfactant reduced the surface tension to values around 31 mN/m and exhibited antimicrobial activity against several pathogenic microorganisms. Furthermore, these biosurfactants are structurally different from those previously reported. The properties exhibited by these biosurfactants, together with their relatively high productivity (108.3 mg/L/h), make them promising candidates for application in several fields, including biomedical applications.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.colsurfb.2017.03.041.

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