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Short communication

Production and characterization of a bioemulsifier from *Yarrowia lipolytica*

P.F.F. Amaral [a], J.M. da Silva [b], M. Lehocky [b], A.M.V. Barros-Timmons [b], M.A.Z. Coelho [a], I.M. Marrucho, J.A.P. Coutinho [b,*]

[a] *Departamento de Eng. Bioquímica, Escola de Química, Universidade Federal do Rio de Janeiro, 21949-900, RJ, Brasil*
[b] *CICECO, Departamento de Química, 3810-193, Universidade de Aveiro, Aveiro, Portugal*

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Abstract

Emulsification activity was identified in the culture medium of a Brazilian wild strain of *Yarrowia lipolytica* IMUFRJ50682 isolated from Guanabara Bay in Rio de Janeiro. The bioemulsifier, secreted during growth in glucose as carbon source, was successfully isolated and characterized. The isolated emulsifier, named Yansan, is different from previous emulsifiers identified for *Y. lipolytica*. It presents high emulsification activity and stability in the pH range of 3.0–9.0 and is capable of stabilizing oil-in-water emulsions with several aliphatic and aromatic hydrocarbons.

Keywords: *Yarrowia lipolytica*; Surfactant; Emulsification activity; Emulsion stability; Hydrophobic substrates; Perfluorodecalin

1. Introduction

Bioemulsifiers are microbial origin compounds with various advantages over synthetic emulsifiers. Their biodegradability is one of their most important assets because it prevents toxicity problems and accumulation in natural ecosystems [1]. Their potential industrial applications include enhanced oil recovery, crude oil drilling, food processing and cosmetic formulations [2]. The ability of bioemulsifiers to emulsify hydrocarbon–water mixtures enhances the degradation of hydrocarbons in the environment.

Bioemulsifier-producing microorganisms can be divided into three categories [3]: those producing bioemulsifiers exclusively with alkanes as carbon source, such as *Corynebacterium* sp.; those producing biosurfactants only with water-soluble substrates as the carbon source, such as *Bacillus* sp.; and those producing biosurfactants with alkanes and water-soluble substrates as carbon sources, such as *Pseudomonas* sp. The production of emulsifying agents from yeast sources usually requires the presence of water-immiscible substrates, which represents a challenge for the isolation of the produced biosurfactant.

Yarrowia lipolytica, a strictly aerobic yeast with the ability to produce a wide spectrum of products, is considered as non-pathogenic and several processes based on this organism were classified as generally recognized as safe (GRAS) by the Food and Drug Administration (FDA, USA) [4]. Moreover, it is known to produce surface-active agents using different substrates [5–7]. A novel bioemulsifier, hereby named Yansan, was isolated from the glucose-based culture medium of *Y. lipolytica* in the absence of any water-immiscible substrates. This bioemulsifier and its production will be hereafter described and characterized.

2. Materials and methods

2.1. Strain, media and culture conditions

A wild type strain of *Yarrowia lipolytica* (IMUFRJ 50682) was employed [8] and kept at 4 °C on YPD-agar medium. For growth conditions, cells were cultivated at 28 °C in a rotary shaker at 250 rpm, in flasks containing YPD medium (w/v: yeast extract (*Oxoid*) 1%; peptone (*Merck*), 0.64%; glucose (*Isofar*), 2%). The assays were performed in 500 mL shake flasks containing 200 mL medium.

2.2. Isolation of the surfactant

The isolation of the surfactant was adapted from the method described by Cirigliano and Carman [5]. The culture was centrifuged (26,000 × *g*) at 25 °C

* Corresponding author. Tel.: +351 234 370 200; fax: +351 234 370 084.
E-mail address: jcoutinho@dq.ua.pt (J.A.P. Coutinho).

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for 20 min and then filtered through a 0.45-μm membrane (Millipore Corp.). Approximately 150 mL of the cell-free filtrate was transferred to a 3.6-ft (110-cm) length of dialysis tubing (diameter, 4 cm; molecular cut-off, 12,000 Da) and concentrated to 25 mL by pervaporation. The concentrated filtrate (25 mL) was extracted with 250 mL of chloroform–methanol (4:1, v/v) in a 500 mL separatory funnel at 25 °C. The white precipitate formed in the aqueous phase was centrifuged (32,000 × *g*) at 4 °C for 30 min, re-suspended in water and lyophilized.

2.3. Characterization of the surfactant

2.3.1. Emulsification activity

The emulsification activity was measured using the assay described by Cirigliano and Carman [5,9]. One unit of emulsification activity was defined as the amount of emulsifier that changed the emulsion absorbance at 540 nm by 1.0 U in the conditions described above [5].

2.3.2. Emulsion stability

To measure the stability of the emulsions formed during the emulsification assay, absorbance was taken every 10 min for 50 min. The log of the absorbance was plotted versus time, and its slope was taken as the decay constant, K_d. To study the effect of pH on the surfactant activity, the assay was performed using sodium acetate buffer at various pH values. The effect of different organic phases on the emulsion stability was studied by replacing the hexadecane in the emulsification assay by 750 mg of aliphatic or aromatic hydrocarbons (HCs) as well as perfluorocarbons (PFCs).

2.3.3. Contact angle measurement

The sample preparation for this measurement followed the method described by Henriques et al. [10]. Two milliliters of an aqueous solution of Yansan (50 mg mL^{-1}) was spread over the solidified agar layer to cover the entire surface, which was dried at room temperature (for 10–15 h). Two more layers were added following the same procedure. Contact angles were measured by the sessile drop technique, using an apparatus OCA 15 Plus, Dataphysics, at room temperature using water, formamide and diiodometane. The angles obtained on the surfactant films were used to calculate the total surface tension (γ^{tot}) and their components: Lifshitz–van der Waals (γ^{LW}) and Lewis acid–base (γ^{AB}) [11]. The free energy of interaction between cells and water (ΔG_{mwm}) was calculated according to van Oss [12].

2.3.4. Critical micellar concentration (CMC)

The critical micellar concentration was determined by turbidimetric titration [13] at 450 nm and by surface tension measurements using the du Nouy ring technique.

2.3.5. Zeta potential

The electrokinetic zeta potential was studied on a Coulter delsa 440SX instrument, with 100 mg of Yansan suspended in 5 mL of an aqueous solution of KCl at ionic strength corresponding to 0.001 M.

2.3.6. Monosaccharide composition

Neutral sugars were determined by gas–liquid chromatography after sulphuric acid hydrolysis [14] and conversion to their alditol acetates [15] in a Carlo Erba 6000 gas chromatograph equipped with a split injector (split ratio 1:60) and a flame ionization detector (FID) detector. The column was a DB-225 (30 m × 0.25 mm, film thickness of 0.25 μm, J&W).

2.3.7. Protein analysis

Protein was estimated by the Folin–Ciocalteau reagent as outlined by Lowry et al. [16], at 500 nm, using bovine serum albumin (BSA) as the standard.

2.3.8. Fatty acids analysis

Before GC–MS analysis, the pre-treatment of the samples was performed as described by Freire et al. [17]. GC–MS analysis were performed using a Trace Gas Chromatograph 2000 Series equipped with a Finnigan Trace MS mass spectrometer, using helium as carrier gas (35 cm s^{-1}), equipped with a DB-1 J&W capillary column (30 m × 0.32 mm i.d., 0.25 μm film thickness).

2.3.9. Chemical characterization

The FT-IR spectrum was recorded using a Mattson 7000 FT-IR. Visible absorption measurements were recorded on a Jasco V560 spectrometer. The FT-Raman spectrum was recorded using a Bruker RFS 100/S spectrophotometer. The NMR spectra were recorded using a Bruker MSL 400 or a DRX 500 MHz Spectrometer.

X-ray photoelectron spectroscopy (XPS) analysis was performed on a bioemulsifier film deposited on a glass slide using ESCALAB 200A, VG Scientific (UK) with PISCES software for data acquisition and analysis. For analysis, an achromatic Al (Kα) X-ray source operating at 15 kV (300 W) was used, and the spectrometer, calibrated with reference to Ag $3d_{5/2}$ (368.27 eV), was operated in CAE mode with 20 eV pass energy. Data acquisition was performed with a pressure lower than 10^{-6} Pa. Spectral analysis was performed using peak fitting with Gaussian–Lorentzian peak shape and Shirley type background subtraction.

A Voyager DE-STR (IBET/GLP224) mass spectrometer (Applied Biosystems) was used for the matrix assisted laser desorption/ionization (MALDI) technique. The sample (Yansan) was tested in MALDI plate with three different matrices: 2,5-dihydroxybenzoic acid (DHB), 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) and a-cyano-4-hydroxycinnamic acid (CHCA).

3. Results and discussion

3.1. Emulsifier production

Y. lipolytica ability to produce emulsifiers induced by the presence of an organic substrate has been described by several authors [5,6,9,19]. Only Sarubbo et al. [7] report the production of emulsifiers with water-soluble carbon sources. In the present work, emulsifying activity was detected in the culture medium of *Y. lipolytica* IMUFRJ 50682 in the presence of glucose as carbon source, while emulsifying activity in Liposan production was only detected when hexadecane was present in the culture medium [5]. The emulsification activity profile from the culture medium of *Y. lipolytica* IMUFRJ 50682 is presented in Fig. 1 showing expressive values when compared to the maximum activity obtained by Cirigliano and Carman [5] for Liposan produced by *Y. lipolytica* in presence of hexadecane (1.2 U of activity). Using the isolation technique described previously the bioemulsifier, herein named Yansan, was obtained as a whitish powder.

Although most surfactant excretion by microorganisms occurs only during the stationary phase [5,6], significant emulsification activity was detected during the exponential phase of growth (Fig. 1), being an advantage as it enhances

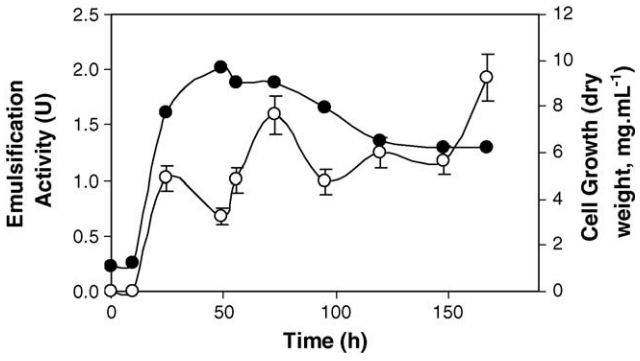

Fig. 1. Emulsification activity production by *Y. lipolytica* grown in YPD medium agitated at 250 rpm. Samples were taken at 12 h intervals and analyzed for emulsification activity (○) and cell growth (●).

productivity. However, Yansan was isolated from culture medium when maximum emulsification activity was detected, 170 h, to maximize the yield.

3.2. Chemical composition

Upon a standard purification step the bioemulsifier was submitted to FT-IR, Raman and ^{1}HNMR analyses for identification of the main functional groups present in Yansan. In the FT-IR spectrum the presence of a large broad band between 3650 and 3200 cm^{-1} was indicative of significant water and O–H content, typical of polysaccharides. The spectrum also showed a strong band at 1654 cm^{-1} (υ C=O, amide), and another intense band at 1060 cm^{-1} (υ C–O–C, ethers). The attribution of the carbonyl band to an amide group was supported by the presence of bands at 1550 cm^{-1} (δ N–H, amide) and at 1410 cm^{-1} (υ C–N, amide). Overall the FT-IR spectrum suggested that the sample is predominantly a polysaccharide although some protein is present. This was confirmed by a ^{1}H NMR spectrum run in D_2O which was dominated by signals attributed to polysaccharide at 3.2–4.4 ppm. The rest of the spectrum indicated the presence of low levels of protein.

Following the method reported by Lowry et al. [16] the protein content was estimated as 15%. This result was confirmed by the nitrogen content determined by X-ray photoelectron spectroscopy analysis (XPS) and elemental analysis (E.A.). The XPS analysis revealed that Yansan contains 62.1% carbon, 7.8% nitrogen, 29.2% oxygen and 0.6% sulfur. The XPS Cx peak was decomposed by a least-square fitting program into three Gaussian components set at 285.0, 286.6 and 288.3 eV by imposing a constant full-width at half maximum of 1.35 eV. These three components had fractions of 0.30, 0.49 and 0.21, respectively, and are representative of carbons involved in C–C and C–H bonds, C–O and C–N bonds, and (C=O)–N and (C=O)–O bonds, respectively. The Ox peak was similarly decomposed into two Gaussian components set at 531.9 and 533.1 eV with fractions of 0.30 and 0.70 representative of the C=O and C–O bonds. The ratio of C=O/C–O bonds is in good agreement in the two peaks. A single N peak for (C=O)–N bonds was found at 163.9 eV and a weak peak for C–S bonds at 164.0 eV was identified.

The lipids content was below the methodology detection limit of 1%. In spite of their low content on the emulsifier it was possible to identify the main fatty acids present as palmitic acid (35.8%), stearic acid (21.4%), lauric acid (8.8%) and oleic acid (6.9%).

The monosaccharides of the isolated bioemulsifier were identified as arabinose, galactose, glucose and mannose (1:6:17:31). Zinjarde et al. [18] reported that the emulsifier produced by *Y. lipolytica* NCIM 3589 has a sugar content of just 20% made up of mannose and galactose without aminosugars present as well. Cirigliano and Carman [9] reported the presence of galactosamine and uronic acids in Liposan, demonstrating significant differences in the sugar composition of the emulsifiers produced by different strains of *Y. lipolytica*.

The molecular weight of this bioemulsifier was assessed by mass spectrometry. The signal was only detected using sinapinic acid, which is the matrix indicated for glycoprotein samples. The mass spectra obtained for Yansan showed a broad peak with a maximum at about 20,000 *m/z*, which is the subunit molecular weight of the complex, much lower than the obtained value for Liposan, estimated to be 27,600 Da [9].

The chemical characterization indicates that the Yansan consists of a polysaccharide–protein complex with low lipid content. Sarrubo et al. [7] produced a bioemulsifier, from *Y. lipolytica* in the presence of glucose as carbon source, which consisted in 47% protein, 45% carbohydrate and 5% lipids. Although produced in the presence of the same carbon source, the biosemulsifier here studied shows a much lower protein and lipid. All the other *Y. lipolytica* emulsifiers were produced with hexadecane as carbon source [5,18].

It has been demonstrated that the protein content of these polymers plays an important role in the emulsification activity [19]. In fact, many mannoproteins, extracted from cell walls of yeasts, have been found to have high emulsification properties [20–22] because the presence of hydrophilic mannose polymers covalently attached to the protein backbone provide the amphiphilic structure common to surface-active agents [21].

3.3. Physico chemical characterization

The critical micelar concentration (CMC) is the minimum emulsifier concentration required for reaching the lowest interfacial or surface tension values [3]. CMC were measured for Yansan and two other natural polysaccharides, Arabic and Xanthan gums, which have emulsification capacities and are commonly used in the food industry. Yansan presents a CMC value (0.50 mg mL^{-1}) inferior to Arabic gum (1.65 mg mL^{-1}) and close to Xanthan gum (0.30 mg mL^{-1}), which means that the amount of Yansan needed to solubilize organic compounds is similar to the amount of to those commercial bioemulsifiers. The surface tension obtained for Yansan at the CMC was 50 mN/m.

The zeta potential of the emulsifier was determined as a function of the pH, as shown in Fig. 2. Yansan micelles present an acidic isoeletric point at pH 2.3 and negative charge around

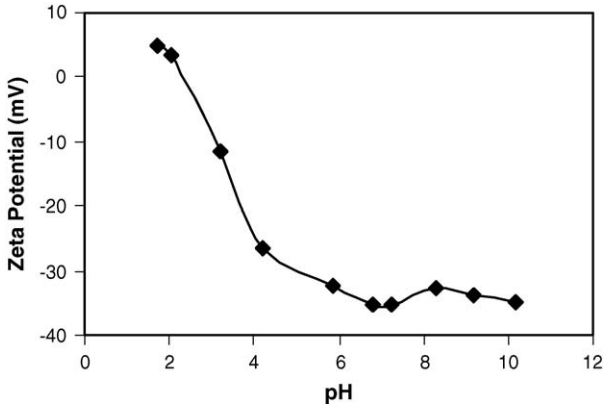

Fig. 2. Zeta potential of Yansan as a function of the medium pH.

−35 mV on its surface for pH values higher than 4. A higher isoeletric point (4.0), but still acidic, was reported for a bioemulsifier produced by *Y. lipolytica* NCIM 5389 [18].

The surface characteristics of Yansan were investigated in terms of their superficial tension components obtained from the contact angle measurements. The apolar surface tension component ($\gamma^{LW} = 14.11$ mN m^{-1}) of Yansan is much higher than its polar component ($\gamma^{AB} = 38.43$ mN m^{-1}), which implies that the hydrophobic part of this amphiphilic molecule is higher than the hydrophilic part. The negative component of the Lewis acid–base component, γ^{AB} ($\gamma^{-} = 43.64$ mN m^{-1}) is much larger than the positive one ($\gamma^{+} = 1.14$ mN m^{-1}), indicating that Yansan surface has an electron-donor character, confirming the results obtained by the zeta potential measurements. The calculated free energy of interaction between Yansan molecules ($\Delta G_{mwm} = 20.11$ mN m^{-1}) is positive, presenting a higher affinity for water than for other Yansan molecules.

3.4. Emulsification activity and emulsion stability

To identify the optimal oil/emulsifier ratio, 30 mg of Yansan were used to emulsify 15–3000 mg of hexadecane. Maximum activity was obtained when the ratio of hexadecane to Yansan was 25:1 (w/w). Further assays were performed using this ratio of HCs or PFCs to emulsifiers.

The pH effect on emulsification activity and stability was studied for Yansan and some commercial surfactants (Pluronic, Span 20, Arabic gum and Tween 80), presented in Fig. 3. Yansan activity is slightly influenced by the pH displaying a shallow maximum between pH 5 and 7. Pluronic and Arabic gum show a tendency for losing activity with increasing pH and present lower emulsification activities than Yansan. Tween 80 is almost not affected by the pH, as expected for a non-ionic surfactant, and for pH values higher than 5, presents an activity very similar to Yansan. These results also show that the decay constant (K_d) for Yansan is higher than for the other surfactants studied, indicating that its emulsions are more stable, and is also fairly pH independent.

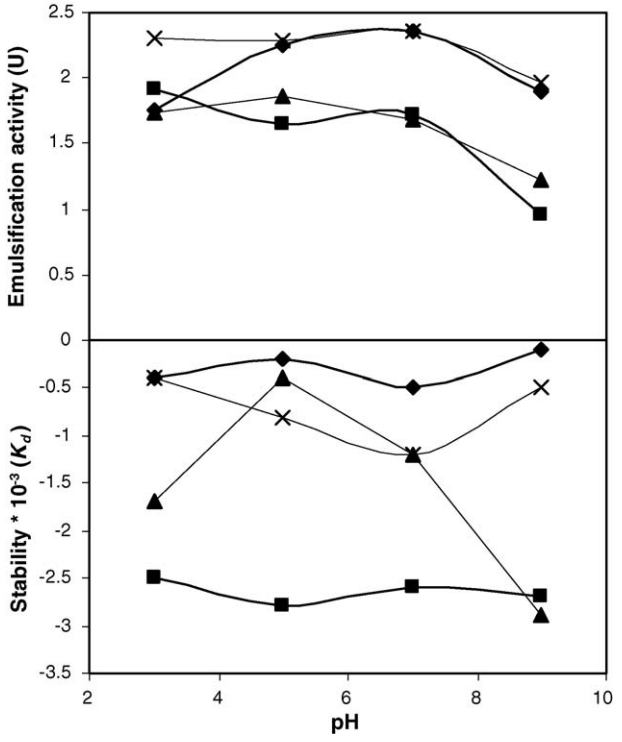

Fig. 3. Effect of pH on emulsification activity and stability of Yansan (◆), Tween 80 (×), Pluronic (▲) and Arabic gum (■).

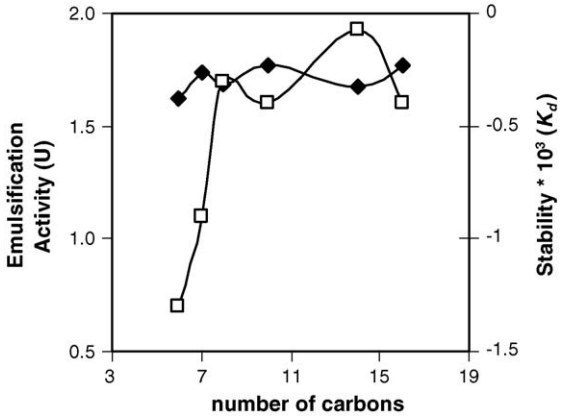

Fig. 4. Effect of carbon chain length of aliphatic hydrocarbons in emulsification activity (◆) and stability (□) of Yansan.

Since Zinjarde et al. [18] reported that the emulsifier produced by *Y. lipolytica* NCIM 3589 was not able to emulsify *n*-alkanes, and since Liposan [5] has shown extremely low emulsification activity with short chain length HCs (from hexane to decane) various aliphatic HCs were analyzed for emulsification activity and stability with Yansan (Fig. 4). Yansan showed an emulsification activity higher than 1.0 U for all the aliphatic HCs tested. The activity increases with the chain length up to octane after which the activity becomes independent of the hydrocarbon size. The activities for paraffinic oils are equivalent to those reported for the higher alkanes (data not shown). The emulsion stability is always very high and independent of the hydrocarbon chain length.

Yansan was capable to form oil-in-water emulsions of aromatic HCs, such as toluene, xylene and styrene, with very

Table 1
Emulsification activity and decay constant for Yansan with aromatic hydrocarbons and perfluorocarbons

	Emulsification activity (U)	Stability × 10^3 (K_d)
Aromatic hydrocarbons		
Toluene	2.63	−0.3
Xylene	2.42	−1.5
Styrene	2.43	−0.08
Perfluorocarbons		
Perfluorooctane	1.82	−0.6
Perfluorononane	1.54	−0.5
Perfluorodecaline	1.15	−0.8
Perfluorotoluene	0.44	−6.9
Hexafluorobenzene	1.68	−2.4

high emulsification activities (Table 1). The emulsification activities are superior to those for Liposan, which presented emulsification activity inferior to 0.75 U with aromatic HCs [5]. The bioemulsifier produced by *Y. lipolytica* NCIM 3589 [18] presented higher emulsification activity towards aromatic HCs. Yansan was capable to form emulsions of both aromatic and aliphatic HCs. The stability of the emulsions formed with Yansan and aromatic hydrocarbons was equivalent to the aliphatic ones. The emulsification activities obtained for the PFCs (Table 1) are equivalent to those reported for alkanes. Unlike HCs no significant difference was observed between aromatic and aliphatic PFCs.

4. Conclusion

A novel bioemulsifier, Yansan, from *Yarrowia lipolytica* IMUFRJ 50682 has been successfully isolated from a glucose-based medium and has been evaluated for its emulsification properties. Yansan is a lipid–carbohydrate–protein complex with high emulsification activity and stability in a wide pH range (3–9) and has the ability to form water-in-oil emulsions with aliphatic or aromatic HCs and with PFCs. This surfactant shows potential application in a variety of fields, especially in biorremediation and formulation of perfluorocarbon-based emulsions [23].

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