CHEMICAL ENGINEERING TRANSACTIONS

VOL. 27, 2012

Guest Editors: Enrico Bardone, Alberto Brucato, Tajalli Keshavarz

ISBN 978-88-95608-18-1; **ISSN** 1974-9791

A publication of AIDIC

The Italian Association of Chemical Engineering
Online at: www.aidic.it/cet

DOI: 10.3303/CET1227012

Properties of the Biosurfactant Produced by *Candida Sphaerica* Cultivated in Low-Cost Substrates

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Many biosurfactants have been produced, although few are produced due to the high costs of production and purification processes. Given the potential for biotechnological application of these compounds in the oil and pharmaceutical industries, the optimization of the use of two industrial wastes, corn steep liquor and ground–nut oil refinery residue e as low cost nutrients for the production of a biosurfactant by *Candida sphaerica* (UCP 0995) was studied. Then the properties of the biosurfactant was described, and its isolation, preliminary chemical characterization. In this paper we used an optimized medium with distilled water supplemented with 9 % ground nut oil refinery residue and 9 % corn steep liquor as substrates to produce biosurfactants by *Candida sphaerica,* at 28°C during 144h under 200rpm. The isolated biosurfactant was formed with a yield of 9g/L. The biosurfactant showed high surface tension reducing activity the 25 mN/m, a small CMC value (0.025 %), thermal (5-120°C) and pH (2-12) stability with respect to surface tension reducing activity and to emulsification activity and tolerance under hight salt concentrations (2-10 %). The biosurfactant was characterized as glycolipid and recovered 95 % of motor oil adsorbed in a sand sample, showing great potential to be used in bioremediation processes, especially in the petroleum industry.

1. Introduction

The industrial need for surfactants is constantlygrowing. Surfactants possess both hydrophilic andhydrophobic structural moieties, which in turn impartmany unusual properties, including an ability tolower the surface tension. Surface-active compoundsproduced by microorganisms are of two main types,those that reduce surface tension at the air–waterinterface (biosurfactants), and those that reduce theinterfacial tension between immiscible liquids, or atthe solid–liquid interface (bioemulsifiers). Biosurfactantsusually exhibit emulsifying capacity but bioemulsifiers do not necessarily reduce the surface tension (Batista et al. 2010). The commercial importance of surfactants is evidenced from the increasing trends in their production and the number of industrial applications (Luna et al. 2011). Their environmental uses are related principally to the bioremediation of petroleum hydrocarbons in groundwater and soil and in the degradation of hazardous compounds (Coimbra et al. 2009). In the oil industry, they are used in microbial-enhanced oil recovery, in the cleaning of contaminated vessels and to facilitate transportation of heavy crude oil by pipeline (Muthusamy et al. 2008). Biosurfactants have been attracting recent attention as natural and promising surfactants because they offer several advantages over chemical surfactants, such as their lower toxicity, their biodegradable nature, and their ecological acceptability (Rufino et al. 2008).

Please cite this article as: Luna J., Rufino R. , Campos-Takaki G. and Sarubbo L., 2012, Properties of the biosurfactant produced by candida sphaerica cultived in low-cost substrates, Chemical Engineering Transactions, 27, 67-72 DOI: 10.3303/CET1227012

Thus, the aim of this work was to produce a biosurfactant from *Candida sphaerica* UCP 0995 cultivated in 9 % ground-nut oil refinery residue and 9% corn steep liquor, and to study the surface and emulsifier activities of this biosurfactant in order to verify its potential for industrial application in the environment.

2. Materials and Methods

2.1 Micro-organism

Candida sphaerica UCP 0995 was obtained from the culture collection of the Catholic University of Pernambuco, Brazil. The micro-organism was maintained at 5ºC on Yeast Mold Agar (YMA) slants containing (w/v): yeast extract (0.3%), malt extract (0.3%), tryptone (0.5%), D-glucose (1.0%) and agar (5.0 %). Transfers were made to fresh agar slants each month to maintain viability.

2.2 Substrates

Two types of industrial waste were used as substrates to produce biosurfactants. Ground-nut oil refinery residue was obtained from ASA LTDA, Recife-PE, Brazil, and corn steep liquor from Corn Products do Brasil, Cabo de Santo Agostinho-PE, Brazil.

2.3 Production of biosurfactant

The production of biosurfactant was performed in distilled water based medium with 9% of refinery residue of soybean oil and 9% of corn steep liquor. The shake flasks were kept under 150 rpm orbital agitation for 144 h at 27ºC.

2.4 Emulsifying activity with different hydrophobic compounds

Emulsification index (EI) was measured using the method described by Cooper and Goldenberg (1987).

2.5 Stability studies

Stability studies were done using the cell-free broth obtained centrifuging the cultures at 5000 g for 20 min. Forty milliliters of the culture broth free of cells were heated at 70, 100 and 120 during 1 h and cooled to room temperature, after which the surface tension and the emulsification index were measured. The surface active properties of culture broth free of cells were also determined after exposure at lower temperature (5°C). To study the pH stability of the cell-free broth, the pH of the cell-free broth was adjusted to different pH values (2.0–12.0) and the surface tension and the emulsification activity were measured. The effect of NaCl concentration (2.0–10.0 %) was also determined in the same way after addition of the salt to the samples. The assays were carried out in triplicate and did not vary more than 5 %.

2.6 Biosurfactant isolation

The culture broth free of cells was acidified with 6 M HCl to pH 2.0 and precipitated with two volumes of methanol. After 24 h at 4°C, samples were centrifuged at 5000 g for 30 min, washed twice with cold methanol and dried at 37°C for 24 to 48 h. The yield in isolated biosurfactant was expressed in g/L. Known amounts of crude precipitate were resuspended in distilled water and used for measurement of the critical micelle concentration (CMC).

2.7 Surface tension and CMC determination

The measurement of the surface tension was carried out on the cell-free broth obtained by centrifuging the cultures at 5000 g for 20 min by the ring method using a Sigma 70 Tensiometer (KSV Instruments Ltd., Finland) at room temperature. The critical micelle concentration (CMC) was determined by measuring the surface tensions of dilutions of isolated biosurfactant in distilled water up to a constant value of surface tension. Each result was the average of 10 determinations after stabilization. The value of CMC was obtained from the plot of surface tension against surfactant concentration.

2.8 Application of the biosurfactant in motor oil removal from contaminated sand

Biosurfactant suitability for enhanced oil recovery was carried through using 60.0 g of beach sand impregnated with 5.0 mL of motor oil. The biosurfactant produced by *C. sphaerica* cultivated in the distilled water supplemented with 9% refinery residue plus 9% corn steep liquor was used in the removal tests. Fractions of 20.0 g of the contaminated Brazilian standard NBR 7214(Brazilian Association of Technical Standards, 1982) and beach sand was transferred to 250 mL Erlenmeyer flasks, which were submitted to the following treatments: addition of 40 mL of the cell-free-broth and addition of 40.0 mL distilled water (control). The samples were incubated on a rotary shaker (150 rpm) for 24 h at 27°C and then were centrifuged at 5000g for 10 min for separation of the laundering solution and the sand. The amount of oil residing in the sand after the action of biosurfactant was gravimetrically determined as the amount of material extracted from the sand by hexane (Luna et al. 2011).

3. Results and Discussion

3.1 Biosurfactant isolation

The yield of the biosurfactant produced by *C. sphaerica* was 9g/L after 144 h of experiment, which is in accordance with the values previously reported in the literature (Luna et al. 2011). Sarubbo et al. (2007) reported a yield of 8g/L for a biosurfactant produced by *C. lipolytica* using canola oil and glucose as substrates

3.2 Surface tension and critical micelle concentration (CMC) of the biosurfactant

The critical micelle concentration (CMC) is the minimum biosurfactant concentration necessary to reduce the surface tension to the maximum extent. The biosurfactant from *C. sphaerica* showed a great surface tension reduction capacity since the water surface tension was reduced from 70 to 25 mN/m with the increase of the biosurfactant concentration up to CMC of 0.25 mg/mL (Figure 1). From this point the increase of biosurfactant concentration did not lead to further reductions in water surface tension, indicating that the CMC had been reached. Results show that the biosurfactant produced by *C. sphaerica* possesses an increased capacity to reduce tension as compared to the biosurfactants from *C. lipolytica* (32 mN/m) (Rufino et al. 2008), *C. glabrata* (31 mN/m) (Sarubbo et al. 2006). Furthermore, the biosurfactant produced in this study also showed a CMC that is much lower than the CMCs reported for other yeast surfactants, considering the rates of 2.5 % for *C. glabrata* (Sarubbo et al. 2007) biosurfactants, 1% for *C. lipolytica* biosurfactant grown in refinery waste (Rufino et al. 2008).

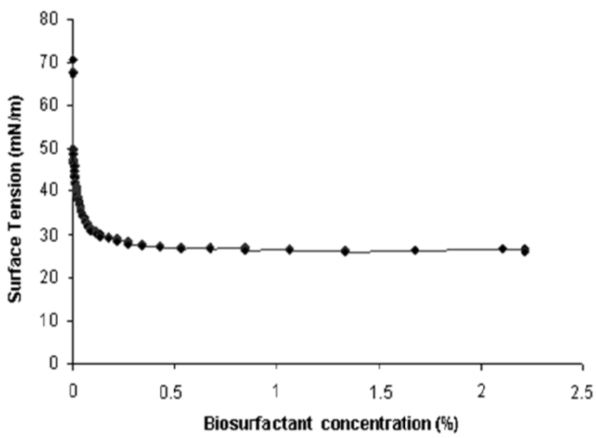

Figure1: Surface tension versus concentration of isolated biosurfactant produced by C. sphaerica grow in distilled water supplemented with 9.0% ground - nut oil refinery residue and 9% corn steep liquor

3.3. Biosurfactant stability related to surface tension and emulsification

The stability of the biosurfactant from *C.sphaerica* was tested over a wide temperature range. The biosurfactant showed to be stable during incubation for 1 h at temperatures ranging from 70, 100 and 120°C (Figure 2A). The surface tension reducing activity and emulsification activity were quite stable at these temperatures and retained practically 100% of the activity against motor oil, at 70^{o}C, 100Cº and 120°C (Figure 3A). The biosurfactant from *C. lipolytica* cultivated in industrial residue (Rufino et al. 2008), and the lipopeptide biosurfactant from *Bacillus subtilis* C9 cultivated in a carbohydrate substrate (Haba et al. 2000), showed similar stability patterns in relation to temperature. Biosurfactant produced by *C. glabrata* (Luna et al. 2009) have shown thermal stability that is similar in connection with the tested temperature range.

Studies on reduction of surface tension by the cell-free-broth containing the biosurfactant showed that it was stable over a wide pH range, and retained practically 100% of the activity against motor oil (Figure2B and Figure 3B). The effectiveness of the biosurfactant from *C. lipolytica* cultivated in canola oil plus glucose as substrates, on the other hand, was limited to the acid to neutral pH range, although an increase in activity was observed under pH 12.0 (Sarubbo et al. 2007).

The resistance of the biosurfactant to NaCl was investigated. The surface tension of the cell-free-broth containing the biosurfactant showed to be stable, independent of the concentration of salt added (Figure 2C). The emulsification activity against motor oil also remained practically unchanged over these tested salt concentrations (Figure 3C). The stability of the biosurfactant from *C. sphaerica* to salinity suggests that it is a good candidate for use in marine environments and some industries related to emulsions. The emulsifier produced by *Candida tropicalis* tolerated 5% sodium chloride and lost 20% of its activity in 10% NaCl (Muthusamy et al.2008).

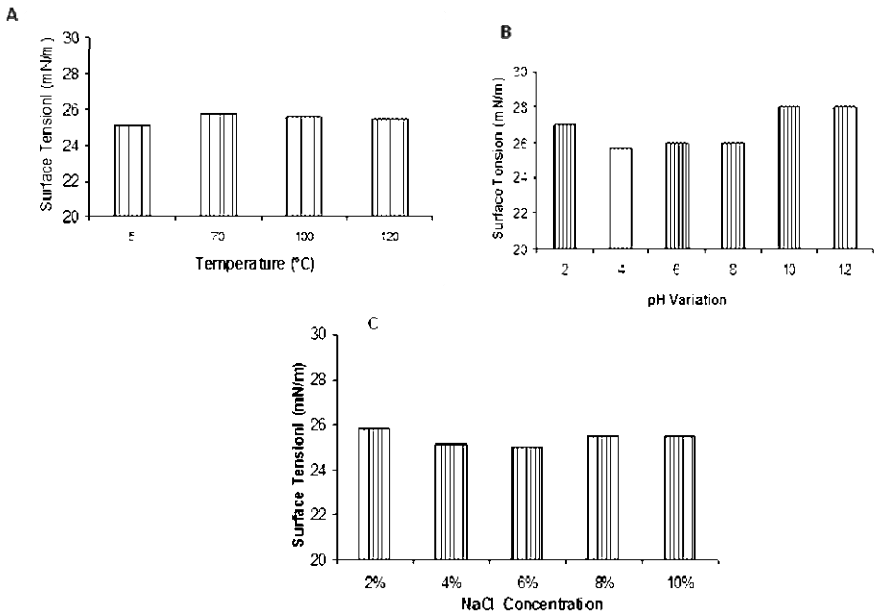

Figure 2-Influence of temperature (A), pH (B) and chloride sodium concentrations (C) on the surface tension of cell-free broth of Candida sphaerica grown medium with 9% of refinery residue of soybean oil and 9% of corn steep liquor

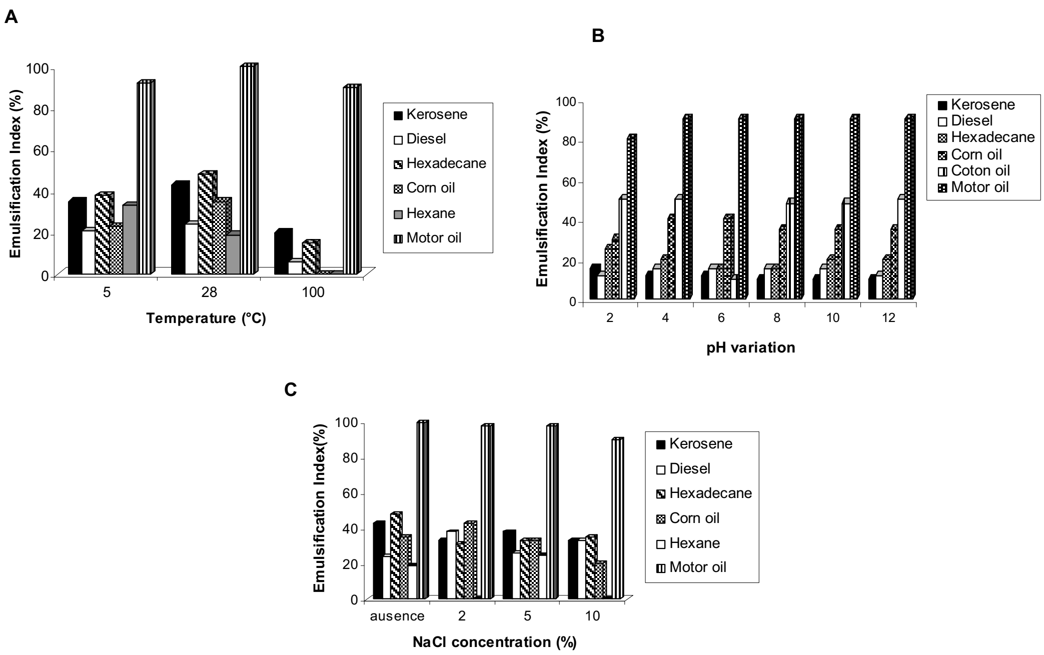

Figure 3- Influence of temperature (A), pH (B) and chloride sodium concentrations (C) on the ulsifying index of cell-free broth of Candida sphaerica grown medium with 9% of refinery residue of soybean oil and 9% of corn steep liquor

3.4 Application of the biosurfactant in the removal of hydrophobic contaminant adsorbed in sand

Biosurfactants can emulsify hydrocarbons enhancing their water solubility, decreasing surface tension and increasing the displacement of oil substances from soil particles (Batista et al. 2010).The results obtained for the removal of motor oil adsorbed in the samples of sand tested by the cell-free culture medium from *C. sphaerica* obtained after 144 h and by distilled water (control) showed the removal of 95 and 10% of the oil, respectively. Once the tests were conducted with the containing biosurfactant cell-free broth (crude biosurfactant), the results were considered satisfactory (Table 1).

Promising results have been obtained by the biosurfactants produced from *Candida* species. Batista et al. (2010), for the cell-free broth containing a biosurfactant produced by *C. tropicallis*, showed the recovery of 80% of residual crude oil adsorbed in the sand and Coimbra et al. (2009), for the biosurfactants produced by *C. guilliermondii* and *C. lipolytica* showed high ability of these molecules in removing motor oil and petroleum adsorbed in the samples of sand tested. Results described in the literature showed that the biosurfactant produced by *C. sphaerica* cultivated in low-cost medium removed 65% of the motor oil adsorbed in beach sand (Sobrinho et al. 2008).

Table 1: *Removal of motor oil adsorbed in beach sand by the cell-free culture medium containing the biosurfactant from C. sphaerica cultivated in and by distilled water (control)*

Sand type	**Contaminant**	**Removal (%)**	
		Cell-free culture medium (crude biosurfactant)	**Distilled water (control)**
Beach sand	**Motor oil**	**95**	**10**

4. Acknowledgements

This work was financially supported by National Council for Scientific and Technological Development (CNPq), Post-Doctoral National Program (PNPD) from Coordination for the Improvement of Higher Level Education Personnel (CAPES), Foundation for the Support of Science and Technology of the State of Pernambuco (FACEPE) and Thermoelectric of Pernambuco (TERMOPE). We are grateful to Center of Sciences and Technology laboratories, from Catholic University of Pernambuco, Brazil.

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