CHEMICAL ENGINEERING TRANSACTIONS
VOL. 49, 2016
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.aidic.it/cet
Guest Editors: Enrico Bardone, Marco Bravi, Tajalli Keshavarz

ISBN 978-88-95608-40-2; **ISSN** 2283-9216
DOI: 10.3303/CET1649098

Production of Biosurfactant from *Candida bombicola* URM 3718 for Environmental Applications

Juliana M. Luna*, Alexandre S. Santos Filho, Raquel D. Rufino, Leonie A. Sarubbo

Centre of Science and Technology, Catholic University of Pernambuco, Rua do Príncipe, n. 526, Boa Vista, Cep: 50050-900, Recife, Pernambuco, Brazil
Julianamouraluna@gmail.com

The processes of bioremediation appear as an innovative technology in the removal of compounds derivate from oil, among others pollutants. Nowadays, it has been given considerable emphasis to the environmental impacts caused by chemical surfactants due to their high toxicity and non-biodegradable properties. Biosurfactants are natural surfactants produced or bacteria, yeasts or fungi from different substrates. Biosurfactants have some advantages over petroleum based surfactants, such as biodegradability, production from renewable substrates, low toxicity, biocompatibility, diversity for chemical structure, effectiveness even at extreme conditions of temperature, pH and salinity. Due to these advantages, biosurfactants are good candidates to be used in environmental applications and in different industries such as petroleum, food production, cosmetics and pharmaceutics. In this work we used a medium formulated with distilled water supplemented with 5% corn steep liquor, 5% molasses and 5% soybean waste frying oil as substrates to produce a biosurfactant from *Candida bombicola,* at 28 °C during 144h under 200rpm. The tensoactive properties of the biosurfactant were determined, and its isolation, preliminary chemical characterization and environmental application were described. The isolated biosurfactant showed a yield of 8.4g/L. The biosurfactant reduced the water surface tension to 30mN/m, with a critical micelle concentration of 0.5%. The biosurfactant demonstrated stability with regard to surface tension reduction in a range of temperatures (5 to 120 ºC) and pH values (2 to 12) as well as tolerance to high concentrations of NaCl (2 to 10%). The biosurfactant was characterized as glycolipid and demonstrated ability to remove 70% of motor oil adsorbed to porous surface. The results obtained with the biosurfactant produced show the promising properties of this biomolecule for use in bioremediation of hydrophobic compounds.

1. Introduction

Surfactants are amphipathic molecules capable of reducing surface and interfacial tensions between liquids, solids and gases (Nitschke and Costa, 2007). All surfactants have two ends, one of which is hydrophobic and the other hydrophilic (Desai and Banat, 1997). A hydrocarbon part usually comprises the hydrophobic end, which is less soluble in water, whereas the water-soluble hydrophilic end may be a carbohydrate, amino acid, cyclic peptide, phosphate, carboxylic acid or alcohol (Rahman, 2008).

The disposal of oil residues from storage, processing and transportation facilities has always been a major issue faced by the petroleum industry. As a result of increasing environmental awareness and the emphasis placed on a sustainable society in harmony with the global environment, natural surfactants of microbial origin have recently been recommended to replace chemically synthesized surface active agents (Luna et. al., 2014). These natural surfactants offer a number of advantages over chemical surfactants, such as adequate inherent biodegradability, low toxicity and ecological acceptability. These substances may also be used at extremes of acidity, temperature and salt concentration and can be produced from in expensive, renewable substrates (Santos et al., 2013; Sarubbo et., 2015).

Biosurfactants are structurally diverse surface active agents produced mainly by hydrocarbon-utilizing bacteria, yeasts and filamentous fungi (Brasileiro et al., 2015). Biosurfactants are utilized in commercial applications in various industries (Banat et al., 2010) and can be classified by their molecular weight a slow molecular mass and high molecular mass polymers. Low-molecular-mass biosurfactants include glycolipids,

Please cite this article as: Luna J.M., Santos Filho A., Rufino R.D., Sarubbo L.A., 2016, Production of a biosurfactant from candida bombicola urm 3718 for environmental applications, Chemical Engineering Transactions, 49, 583-588 DOI: 10.3303/CET1649098

lipopeptides and phospholipids, while high-molecular- mass biosurfactants include polymeric and particulate surfactants and may act as emulsion stabilizing agents (Nitschke and Costa, 2007). Some of the most common biosurfactants are glycolipids, rhamnolipids, sophorolipids, trehalolipids, lipoproteins and lipo peptides, fatty acids, phospholipids and polymeric structures such as emulsan and liposan (Chrzanowski et al., 2012).

Thus, the aim of this work was to produce a biosurfactant from *Candida bombicola* URM 3718 cultivated in 5% corn steep liquor, 5% molasses and 5% soybean waste frying oil and to study the properties of the biosurfactant under extreme environmental conditions, its isolation, preliminary chemical characterization in order to verify its potential for industrial application in the environment.

2. Materials and Methods

2.1 Micro-organism

Candida bombicola URM 3718 was obtained from the culture collection of the Federal University of Pernambuco, Brazil. The micro-organism was maintained at 5 ºC 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

Industrial waste were used as substrates to produce the biosurfactant. Corn steep liquor was obtained from “Corn Products do Brasil” in the municipality of Cabo de Santo Agostinho, state of Pernambuco, Brazil. Sugarcane molasses were obtained from the São José sugar processing plant in the municipality of Cabo de Santo Agostinho, state of Pernambuco, Brazil, waste frying oil obtained from a local restaurant in the city of Recife, state of Pernambuco, Brazil, stored according to the supplier's recommendations and used without any further processing.

2.3 Production of biosurfactant

The production of biosurfactant was performed in distilled water based medium with 5% corn steep liquor, 5% molasses and 5% soybean waste frying oil. The shake flasks were kept under 150 rpm orbital agitation for 120 h at 28 ºC.

2.4 Stability studies

Stability studies were done using the cell-free broth obtained centrifuging the cultures at 5000 g for 20 min. 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 %.

2.5 Biosurfactant isolation

The culture broth free of cells was acidified with 6 MHCl 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).

2.6 Determination of surface tension and CMC

The determination of surface tension was carried out in the cell-free broth obtained by centrifuging the cultures at 5000 x g for 20 min at room temperature using a Sigma 700 digital surface tensiometer (KSV Instruments LTD - Finland), working on the principle of the Du Nuoy ring method. The CMC was determined by measuring the surface tension of dilutions of the isolated biosurfactant in distilled water up to a constant value of surface tension. Stabilisation was allowed to occur until the standard deviation of 10 successive measurements was less than 0.4 mN/m. Each result was the mean of 10 determinations after stabilisation. The CMC value was obtained from the plot of the surface tension against the surfactant concentration and was determined as g/l of biosurfactant.

2.7 Biosurfactant composition

The protein concentration in the isolated biosurfactant was determined using the method developed by Lowry et al. (1951), with bovine albumin as the standard. Total carbohydrate content was determined using the phenol-sulphuric acid method (Hanson et al., 1981) and lipids were quantified based on Manocha et al. (1980): 0.5 g of the isolated material were extracted with chloroform: methanol at different proportions (1:1 and

1:2, v/v). The organic extracts were then evaporated under vacuum conditions and the lipid content was determined by gravimetric estimation.

2.8 Determination of ionic character

The ionic charge of the biosurfactant was determined using the agar double diffusion technique (Silva et al., 2010). Two regularly spaced rows of wells were made in an agar of low hardness (1% agar). The wells in one row were filled with the biosurfactant solution and those of the other side were filled a pure compound of known ionic charge. The anionic substance chosen was sodium dodecyl sulphate (SDS) 20 Mm and the cationic substance was barium chloride 50 mM. The appearance of precipitation lines between the wells (indicative of the ionic character of the biosurfactant) was monitored over a 48 h period at room temperature.

2.9 Application of biosurfactant in dispersing hydrophobic contaminant in sea water

The oil displacement test was carried out by slowly dropping 15 µL of motor oil onto the surface of 40 mL of distilled water in a Petri dish (15 cm in diameter) until covering the entire surface area of the water. This was followed by the addition of 10 µL of the cell-free metabolic broth (crude biosurfactant) and the isolated biosurfactant concentrations: at 0.5 % concentration (at the CMC) and cell free bhoth onto the surface of the oil layer. The mean diameter of the clear zones of triplicate experiments was measured and calculated as the rate of the Petri dish diameter (Ohno et al., 1993).

2.10 Washing of hydrophobic compound adsorbed to porous surface

The removal of motor oil adsorbed to rock was carried out by soaking the material in the contaminant until complete coverage and recording the volume spent. The material was then carefully placed in a 100 ml beaker with the aid of a pincers and submitted to washing with the cell-free metabolic broth (crude biosurfactant) and with the isolated biosurfactant at CMC concentration. After the culture process, the percentage of removal through washing was calculated. The amount of oil remaining on the washed solid was determined by gravimetry (Silva et al., 2010).

3. Results and Discussion

3.1 Biosurfactant stability related to surface tension

An emulsion is formed when one liquid phase is dispersed as microscopic droplets in another (continuous) liquid phase. Most microbial surfactants are substrate specific and solubilise or emulsify different hydrocarbons at different rates (Illori et al., 2005). The poor emulsification of some hydrocarbons may be due to the inability of the biosurfactant to stabilise the microscopic droplets. Environmental factors, such as pH, salinity and temperature, also affect the activity and stability of biosurfactants and it is therefore important to study the influence of these parameters when considering specific applications for these compounds (Mulligan, 2005).

In the present study, the reduction in surface tension by the cell-free broth containing the biosurfactant demonstrated stability at different pH values (Figure 1A). The surface tension of the cell-free broth of biosurfactants isolated from *Bacillus strains* cultivated in molasses and cheese whey was found to remain unaltered when submitted to the same pH range studied in the present investigation (Josi et al., 2008).

The resistance of the biosurfactant to NaCl was investigated. The surface tension of the cell-free broth containing the biosurfactant proved stable, independent of the concentration of salt added (Figure 1B)

The biosurfactant he surface tension of the cell-free broth containing the biosurfactant remained practically unchanged over of temperature tested (Figure 1C).

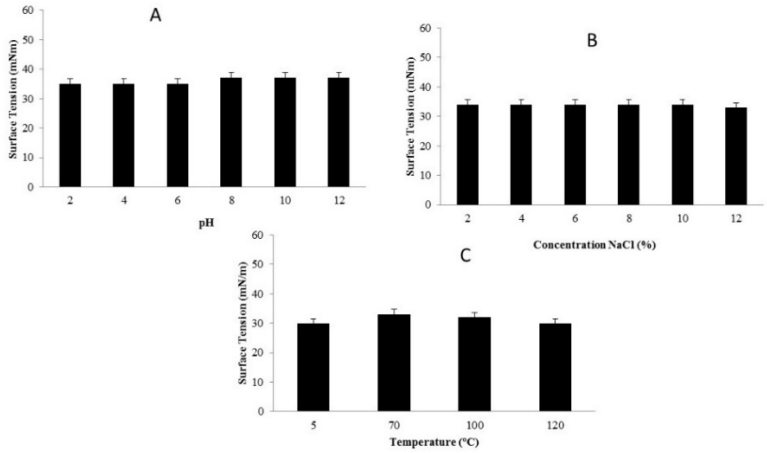

Figure 1: Influence of pH (A), NaCl (B) and temperature (C) on surface tension-reduction capacity of biosurfactant produced by C. bombicola grown in distilled water supplemented with 5% corn steep liquor, 5% molasses and 5% soybean waste frying as substrates. Error bars illustrate experimental errors (standard deviations), calculated from two independent experiments.

3.2 Surface tension and CMC of biosurfactant

The CMC is the minimal concentration of biosurfactant necessary to reduce the surface tension to the maximal extent. The biosurfactant from *C. bombicola* demonstrated an excellent capacity to reduce surface tension. The water surface tension was reduced from 70 to 30 mN/m with the increase in the concentration of the biosurfactant of up to 0.5% (Figure 2), at which point, an increase in biosurfactant concentration did not lead to a further reduction in water surface tension, indicating that the CMC had been reached. These results demonstrate that the biosurfactant produced by *C. sphaerica* has a greater capacity to reduce tension in comparison to biosurfactants from *C. lipolytica* (32 mN/m) (Rufino et al., 2008), *C. glabrata* (31 mN/m) (Sarubbo et al., 2006), *C. antarctica* (35 mN/m) (Gallert and Winter, 2002) and *Y. lipolytica* (50 mN/m) (Shepherd and Rockey, 2002). Furthermore, the biosurfactant produced in the present study also has a much lower CMC than that reported for other yeast surfactants, considering rates of 2.5% for a biosurfactant from *C. glabrata* (Sarubbo et al., 2006), 1% for a biosurfactant from *C. lipolytica* grown in refinery waste (Rufino et al., 2008) and 0.8% for a biosurfactant from *C. sphaerica* (Sobrinho et al., 2008).

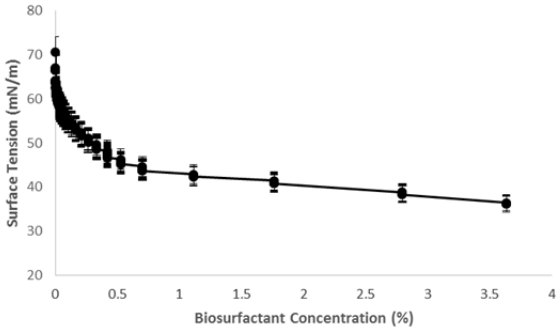

Figure 2: Critical micelle concentration of biosurfactant produced by C. bombicola grown in distilled water supplemented with 5% corn steep liquor, 5% molasses and 5% soybean waste frying as substrates. Error bars illustrate experimental errors (standard deviations), calculated from two independent experiments.

3.3 Preliminary characterisation of biosurfactant

The preliminary analysis demonstrated that the biosurfactant isolated from *C. bombicola* consisting of 70% lipids, 10% carbohydrates and 20% proteins. There are numerous reports on the isolation and surfactant production of different species of the genus *Candida*. The surfactants produced by this genus can differ widely from one species to another, with reports of sophorose lipids (Shepherd and Rockey, 2002), lipid-carbohydrate complexes (Gallert and Winter, 2002), protein-carbohydrate complexes (Cirigliano and Carman,

1984), long-chain fatty acids (Kappeli et al., 1978) when grown on either hydrophobic or water-miscible substrates.

3.4 Determination of ionic character

The agar double diffusion tests revealed the appearance of precipitation lines between the biosurfactant produced by *C. bombicola* and the cationic compound used (barium chloride), while no lines formed between the biosurfactant and the anionic compound (SDS). Under the experimental conditions of the present study, this simple test demonstrated the anionic character of the biosurfactant produced. Other biosurfactants produced by *Candida* species also display an anionic character when submitted to the same test (Sobrinho et al., 2008)

3.5 Application of the biosurfactant in removal of hydrophobic compound to porous surface

The removal of lubricating motor oil and petroleum impregnated in porous surface was carried out by using 100 mL aqueous solutions of the isolated biosurfactant from *C. bombicola* at the following concentrations: at 0.5% concentration (at the CMC), 1.0 % and cell free broth, the contaminant not removed was determined in the washed coral reef by gravimetric assay after extraction with hexane and expressed in percentage.

3.6 Application of biosurfactant in dispersing hydrophobic contaminant in sea water

The biosurfactant produced by *C. bombicola* showed high dispersant activity for the lubricating oil of car engine, which could facilitate the targeting of oily spots in the ocean. The cell-free broth (crude biosurfactant) achieved a dispersion rate of 80 % of the initial diameter of the oil, whereas the dispersion rate obtained with the isolated biosurfactant was 50 %. The results were observed when low amounts of biosurfactant were used, thus demonstrating the potential of these compounds in transporting and solubilizing oil spots on marine aquatic environment (Figure 3).

Figure 3: Illustration of motor oil drop before (A) and after (B) dispersion due to the action of biosurfactant produced by C. bombicola grown in distilled water supplemented with 5% corn steep liquor, 5% molasses and 5% soybean waste frying as substrates.

4. Conclusion

The biosurfactant produced has potential for application in the remediation of water contaminated with oil. It is important to note that the best results were obtained with the crude biosurfactant, which represents a considerable reduction of the production costs of this compound, increasing the chances of an actual industrial application.

Acknowledgments

This work was financially supported by Foundation for the Support of Science and Technology of the State of Pernambuco (FACEPE). We are grateful to Center of Sciences and Technology laboratories, from Catholic University of Pernambuco, Brazil.

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