CHEMICAL ENGINEERING TRANSACTIONS
VOL. 64, 2018
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.aidic.it/cet
Guest Editors: Enrico Bardone, Antonio Marzocchella, Tajalli Keshavarz

ISBN 978-88-95608-56-3; **ISSN** 2283-9216
DOI: 10.3303/CET1864100

Production in Bioreactor, Toxicity and Stability of a Low-cost Biosurfactant

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Biosurfactants are natural surfactants produced by bacteria, yeasts or fungi from different substrates, including sugars, oils, and alkanes. Biosurfactants are expected to reach more than USD 2 billion by 2020, with industrial applications in microbial enhanced oil recovery (MEOR), removal of contamination by heavy metals, bioremediation, food, cosmetics, pharmaceuticals and biomedicine. The considerable interest in these biobased products is related to their properties, as biodegradability, production from renewable substrates, low toxicity and biocompatibility, diversity for chemical structure and properties, effectiveness even at extreme conditions of temperature, pH and salinity. Despite the advantages, fermentation must be cost-competitive with chemical synthesis, and many of the potential applications that have been considered for biosurfactants depend on whether they can be produced economically. Fermentation medium can represent approximately 30 % of the cost for a microbial fermentation. In this work we used a medium formulated with distilled water supplemented with 5 % (v/v) corn steep liquor, 5 % (v/v) molasses and 5 % (v/v) soybean waste frying oil as substrates to produce a biosurfactant from *Candida bombicola*, at 28 °C during 120 h under 200 rpm was first produced in 3.0 L bioreactor. The properties of the biosurfactant, toxicity and environmental application were determined. The isolated biosurfactant showed a yield of 61 g/L. The biosurfactant with a critical micelle concentration of 0.5 % and demonstrated low toxicity to the vegetables. The biosurfactant demonstrated stability with regard to emulsification and surface tension reduction in a range of temperatures (4 to 120 ºC) and pH values (2 to 12) as well as tolerance to high concentrations of NaCl (2 to 10 %). The cell-free broth was also effective in oil displacement (90 %) in water. The results obtained with the biosurfactant produced show the promising properties of this biomolecule for use in bioremediation of hydrophobic compounds.

1. Introduction

Surface-active compounds produced by microorganisms are known as biosurfactants. These have, in particular, emulsifying, stabilizing and thickening properties, in most cases presenting complex molecular structures including lipopeptides, glycolipids, fatty acids and phospholipids. Furthermore, they are biodegradable and have low toxicity (Santos et al., 2016).
The genus Candida has been prominent in the production of biosurfactants, either for potential industrial use (Bourdichon et al., 2012) or in the use of industrial residues for optimization in the production of them (Sarubbo et al., 2006, 2007; Santos et al., 2014; Gao et al., 2013; Rufino et al., 2008; Campos et al., 2013; Luna et al., 2013; Sobrinho et al., 2013; Rocha e Silva et al., 2014).

Please cite this article as: Pinto M.I., Ribeiro B., Guerra J.M.C., Rufino R., Sarubbo L., Dos Santos V. A., Luna J., 2018, Production in bioreactor, toxicity and stability of a low-cost biosurfactant, Chemical Engineering Transactions, 64, 595-600 DOI: 10.3303/CET1864100

Because of their amphiphilic structure, biosurfactants increase the surface area of water-insoluble substances by increasing the bioavailability of such substances in water and altering the surface properties of the bacterial cell by making the surfactants excellent emulsifiers, foaming agents and dispersing agents. Compared with their chemically synthesized equivalents, they have many advantages because they are ecologically correct, biodegradable, less toxic and non-hazardous (Muthusamy et al., 2008). They can be produced from industrial wastes and by-products making the production of biosurfactants possible in addition to allowing the use of waste substrates and, at the same time, reducing their pollutant effect (Das et al., 2008).
The possibility of producing biosurfactants from renewable substrates and different microbial species, besides the possibility of varying cultural parameters such as cultivation time, stirring speed, medium pH and added nutrients, allows to obtain compounds with structural characteristics and different physical properties, which make them comparable or superior to synthetic surfactants in terms of efficiency, although the production costs still do not allow a greater competitiveness with the synthetic (Coimbra et al., 2009; Sarubbo et al., 2015). In this sense, the objective of this work was to produce in a bioreactor a low cost biosurfactant from *Candida bombicola* yeast evaluating toxicological risks and stability.

2. Material and Methods

2.1 Microrganisms

The yeast *Candida bombicola*, maintained in YMA (Yeast Mold Agar) was used as a microorganism producing the biosurfactant. Repiques were monthly performed to maintain cell viability.

2.2. Means of Maintenance

Cultures were maintained at 5ºC in Yeast Mold Agar (YMA) medium with the following composition (w/v): yeast extract (0.3 %), malt extract (0.3 %), tryptone (0.5 %), D -glucose (1 %) and agar (5 %). Monthly transfers were made to fresh slants to maintain cell viability.

2.3. Inoculum growth medium

The growth of the inoculum was carried out in YMB (Yeast Mold Broth) medium, which has the same composition as the YMA medium, excluding agar.

2.4. Production medium

The fermentations for the production of the biosurfactant were carried out in a medium formulated with distilled water containing 5 % (v/v) of sugar cane molasses, 5 % (v/v) of residual frying oil and 5 % (v/v) of corn steep liquor.

2.5. Preparation of inoculum

The inoculum was standardized by transferring the yeast into a tube containing the YMA medium in order to obtain a youthful culture. The sample was then transferred to vials containing 50 mL of the YMB medium and then incubated under 200 rpm shaking at 28 °C for 24 hours. After this time, dilutions were performed until the desired final cell concentration (10^6 cells / mL) was achieved.

2.6. Production of biosurfactant

The fermentations for the production of the biosurfactant were carried out in 3.0 L bioreactor capacity containing 1.5 L of the production medium and incubated with the cell suspension of 10^6 cells / ml. The bioreactor was maintained under orbital stirring at 200 rpm for 120 hours at 28 °C. Aliquots were collected after fermentation to determine surface tension.

2.7. Determination of emulsification activity

For the determination of the emulsification activity, the samples were centrifuged at 4500 g for 15 minutes and then analyzed according to the methodology proposed by Cooper and Goldenberg (1987). Where by 2ml of a liquid hydrophobic compound (canola oil, corn oil and soy oil) was added to 2ml of the culture broth free of cells in a graduated screwcap test tube, and vortexed at high speed for 2min. The emulsion stability was determined after 24 h and the emulsification index was calculated by dividing the measured height of the emulsion layer by the mixture's total height and multiplying by 100.

2.8. Determination of surface tension

The surface tension was measured using a KSV Sigma 70 (Finland) automatic tensiometer using the NUOY ring technique.

2.9. Isolation of biosurfactant

The metabolic liquid was extracted using ethyl acetate repeating the procedure three times. Then the solvent was transferred to a separatory funnel, discarding the aqueous phase and the solvent phase was dried using sodium sulfate and then filtered and evaporated.

2.10. Phytotoxicity test

The phytotoxicity of the biosurfactant was evaluated in a static assay by seed germination and root growth of Solanum lycopersicum (tomato) and *Cucumis anguria* (maxixe), according to Tiquia et al. (1996). Test solutions of the isolated biosurfactant were prepared in distilled water at different concentrations (1/2xCMC, CMC and 2XCMC). After five days of incubation in the dark, seed germination, root growth (≥ 5 mm) and germination index (GI) were calculated according to the formulas below:

Relative germination of the seed (%) = (number of seeds germinated in the extract / number of seeds germinated in the control) x 100

Relative root length (%) = (mean root length in extract / mean root length in control) x 100

GI = [(% seed germination) x (% root growth)] / 100 %

2.11. Chemical and physical stability of the emulsion

The emulsion stability of the biosurfactant produced was determined by the determination of the emulsification activity produced at different pHs (2,4,6,8,12 and 12), variable saline (NaCl) concentrations (2,4,6,8 and 10 %), different heating intervals (15 to 180 minutes in autoclave at 120 ° C) and different temperatures (4, 25, 55 and 85 ° C). The surface tension was measured on a KSV Sigma 700 (Finland) automatic tensiometer using the NUOY ring technique.

2.12. Application of biosurfactant in the dispersion of hydrophobic contaminant in sea water

The oil displacement test was performed by the slow drop of 15 µL of motor oil on the surface of 40 mL of sea water in a Petri dish (14 cm in diameter). This was followed by the addition of 10 µl of the biosurfactant alone at concentrations at 0.25 %, 0.5 % and 1.0 % concentration. The mean diameter of the clear zones of triplicate experiments was measured and calculated as the Petri dish diameter rate (Ohno et al., 1993).

3. Results and discussion

3.1. Determination of the properties of biosurfactant produced in shaker and bioreactor

The reduction of surface or interfacial tension is considered the main parameter for the detection of a surfactant compound in a given medium (Luna et al., 2015). Table 1 shows the study of the properties of biosurfactant produced in 3.0 L bioreactor. The biosurfactant of *Candida bombicola* exhibited excellent surface tension reduction ability, since the surface tension of the water was reduced from 70 mN / m to 39 mN / m in 3.0 L bioreactor. Regarding the emulsification activity, it was observed that the result is very similar among all the oils obtaining on average 30 % emulsification. It was observed that the yield obtained using ethyl acetate was 61 g / L of biosurfactant. Compared with the results observed in the literature by Almeida et al. (2016) where the biosurfactant produced by *C. tropicallis* grown in medium containing 2.5 % (v/v) molasses, 2.5 % (v/v) residual canola oil had a surface tension of 29.52 mN / m in a yield of 7.0 g / L. While Luna et. al. (2013), observed that the biosurfactant produced by *C. sphaerica* in medium containing 9.0 % (w/v) soybean oil and 9.0 %(v/v) had a surface tension of 25 mN / m with a yield of 8.0 g / L.

Table 1: Study of the production of biosurfactant produced in 3 L bioreactor and with evaluation of surface tension, emulsification index and yield.

Production of biosurfactant	**Surface Tension** (mN/m)	**Emulsification Index** (%)			**Yield** (g/L)
		Canola Oil	**Corn Oil**	**Soy Oil**	
Bioreactor 3.0 L	39.0	28	26	30	61.0

3.2. Toxicity test with tomato and maxixe

The germination index (GI), which combines the measures of relative seed germination and relative root growth, was used to evaluate the biosurfactant toxicity against tomato (*Solanum lycopersicum*) and maxixe (*Cucumis anguria*). The results indicated that the solutions tested did not present inhibitory effect on seed germination and on the elongation of tomato and maxixe roots, since IG of 64 %, 45 % and 0 % for tomato, and of 41 %, 28 % and 0 % for maxixe were obtained for the biosurfactant solutions at the concentrations of 1/2CMC, CMC and 2xCMC, respectively.

Ecotoxicity tests were conducted by Franzetti et al. (2012) for the polysaccharidic bioemulsifiers produced by *V. paradoxus* 7bCT5. Acute toxicity test on crustaceans showed a 100% survival of the organisms. The Germination Indexes calculated from seed germination and root elongation tests did not significantly differ from their controls. Therefore, aqueous solutions of the bioemulsifiers did not result in toxicity to any tested organisms (bacteria, crustaceans or plants). The same solution was used for contact tests on earthworms, showing 100% of survival.

Table 2: Influence of different heating intervals, pH, temperature and salt concentration on surface tension-reduction capacity of biosurfactant produced by C. bombicola. Experiments were performed in duplicate and the results represent the mean ± standard deviation of the two independent experiments.

Parameters evaluated	Surface Tension (mN/m)
Different heating intervals at 121 ºC (min)	
15	33.51 ± 0.09
30	33.68 ± 0.04
60	33.23 ± 0.04
120	32.89 ± 0.06
180	32.93 ± 0.02
Temperature variation (ºC)	
4.0	33.67 ± 0.01
25	33.97 ± 0.01
55	34.06 ± 0.01
85	33.96 ± 0.01
Saline concentration (%)	
2.0	34.26 ± 0.02
4.0	34.25 ± 0.18
6.0	34.00 ± 0.06
8.0	33.70 ± 0.02
10	33.39 ± 0.02
pH variation	
2.0	35.34 ± 0.03
4.0	34.46 ± 0.02
6.0	31.78 ± 0.07
8.0	37.11 ± 0.20
10.0	34.74 ± 0.04
12.0	35.53 ± 0.16

3.3. Stability

The stability of the biosurfactant was evaluated against variations in ionic strength, time, pH and temperature, the results of which are described in Table 2. Analyzing the thermal stability results, it was possible to observe that there was no significant change in the surface activity of the biosurfactant, presenting stable against thermal stress conditions. These results are similar to the results presented by Al-bahry et al. (2013). In

relation to the effects of heat treatment at various time intervals and temperatures, Dubey et al. (2012) observed that biosurfactant produced by *P. aeruginosa* PP2 and *Kocuria turfansis* J were thermostable up to 121 ° C and 60 ° C, respectively. When observed in front of the ionic strength, the biosurfactant presented stability for all saline concentrations studied, being possible to use it in marine environments and in other systems where the salt concentration is higher than the physiological level. Recent studies have shown that *C. utilis* (Campos et al., 2014), *C. sphaerica* (Luna et al., 2013) and *C. lipolytica* (Santos et al., 2014) are stable in the presence of various salt concentrations.

Comparing the surface tension results in the study of pH variation, it was observed that the lowest values, 34.46 mN / m and 31.78 mN / m, correspond to pHs 4 and 6, respectively, being close to the pH value of the solution containing the biosurfactant studied (pH 4.5). The variation of the surface tension results along the pH scale is also in agreement with Al-bahry et al. (2013), which can be justified by the fact that acid and basic media influence the solubility of the biosurfactant, their ability to reduce surface tension.

3.4. Application of biosurfactant in the dispersion of hydrophobic contaminant in sea water

The biosurfactant produced by *C. bombicola* showed high dispersant activity for the lubricating oil, which could facilitate the targeting of oily spots in the ocean. The cell-free broth (crude biosurfactant) achieved a dispersion rate of 90 % of the initial diameter of the oil, whereas the dispersion rate obtained with the isolated biosurfactant was 40 %, 50 % and 90 % dispersion of the oily compound in the water after the addition of the solution with the biosurfactant in the concentrations of ½xCMC, CMC and 2xCMC, respectively. 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.

4. Conclusions

The medium formulated with agroindustrial residues presents importance to the process, since it collaborates with the reduction of production costs related to the substrates. The results obtained for the production of a biosurfactant by *Candida bombicola* demonstrate that the biomolecule presents promising properties with regard to surface tension and emulsification index. The biosurfactant maintained its stability in the extreme conditions of pH, salinity and temperature, as well as low toxicity to plant seeds, showing potential for industrial application.

Acknowledgments

This study was funded by the Research and Development Program from National Agency of Electrical Energy (ANEEL) and Thermoelectric EPASA (Centrais Elétricas da Paraíba), the Foundation for the Support of Science and Technology of the State of Pernambuco (FACEPE), the National Council for Scientific and Technological Development (CNPq), and the Coordination for the Improvement of Higher Level Education Personnel (CAPES).The authors are grateful to the Centre of Sciences and Technology of the Universidade Católica de Pernambuco and to the Advanced Institute of Technology and Innovation (IATI), Brazil.

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