CHEMICAL ENGINEERING TRANSACTIONS
VOL. 100, 2023
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.cetjournal.it
Guest Editors: Sauro Pierucci, Flavio Manenti

ISBN 979-12-81206-03-8; **ISSN** 2283-9216
DOI: 10.3303/CET23100015

Production of the Biosurfactent C*andida Guilliermondii* and Application in the Formulation of a Natural Detergent

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Surfactants are molecules that reduce surface and interfacial tension, providing properties such as detergency, emulsification and phase dispersion. Most surfactants in use are derived from petroleum; however, interest in microbiological surfactants has increased due to their biodegradability, reduced toxicity and potential application in several areas of the industrial and environmental sectors. Therefore, in the present study, the biotechnological potential of the yeast *Candida guilliermondii* UCP 0992 was investigated in relation to the production of biosurfactant using different combinations of three compounds (cotton oil, sucrose and glucose). These parameters were used to select the best medium to be reproduced in a 5 L bioreactor at 30 ºC under agitation at 350 rpm and aeration at 0.5 vvm for 192 hours. The metabolic liquid produced in the bioreactor was subjected to determination of surface tension, isolation, toxicity and application in the formulation of a natural detergent for removing dirt. The best result for obtaining the biosurfactant was in the medium with cotton oil and glucose at pH 5.0, with a surface tension of 32.18 mN.m-1, meaning a 55% reduction in relation to the tension of distilled water (72 mN/m), with emulsification activity values between 40 and 78%. The extraction of biosurfactants from *Candida guilliermondii* UCP 0992 in solvents (ethyl acetate and isopropanol) yielded yields of 21 gL^{-1}, with Critical Micellar Concentration (CMC) values of 0.72 gL^{-1}. The biosurfactant also showed no toxicity and was used in the formulation of a commercial detergent, which was applied in tests to remove dirt from cotton fabric, demonstrating efficiency in the immediate removal of contaminants (post-use engine oil, soy oil and automotive grease). Thus, the results point to a potential application of the biosurfactant from the strain *Candida guilliermondii* UCP 0992 in the formulation of biodegradable and non-toxic commercial detergents.

1. Introduction

Environmental legislation and worldwide governmental law restrictions related to the use of toxic detergents in commercialized products have led to a greater interest in the development and use of biodetergents, or biosurfactants, as they are scientifically known, as possible alternatives to chemical surfactants. Currently, biosurfactants are commonly considered as the next generation of industrial surfactants, because they meet most of the necessary requirements for an industrial project with low environmental impact (Sales da Silva et al., 2020). Biosurfactants are amphiphilic molecules produced by microorganisms (yeasts, bacteria and fungi) from renewable resources such as carbohydrates and natural oils, and are considered ecological due to their high biodegradability and reduced toxicity (Selva Filho et al., 2023). They have been effectively tested in a wide range of applications such as oil recovery, soil and water bioremediation, oil mobilization, pharmaceutical preparations, cleaning product formulations and the production of food emulsifiers, among others (Sarubbo et al., 2022). Recent studies on their properties and the scale-up of production for industrial use are undoubtedly some of the main reasons behind the

Paper Received: 17 December 2022; Revised: 20 March 2023; Accepted: 4 June 2023
Please cite this article as: de Souza C.V.M., Torres Jara A.M.A., Bezerra K.G., da Silva I.A., de Santana Costa A.F., de Almeida F.C., Sarubbo L.A., 2023, Evaluation of the Environmental Toxicity Profile of Shampoos Formulated with Plant Biosurfactants /Production of the Biosurfactent Candida Guilliermondii and Application in the Formulation of a Natural Detergent, Chemical Engineering Transactions, 100, 85-90 DOI:10.3303/CET23100015

success of these compounds. Among the various types of microorganisms used in the production of these biosurfactants, yeast stands out, capable of producing large amounts of biosurfactants (on average 300 g/L) to meet a possible industrial demand (Van Bogaert et al., 2019; Farias et al., 2021).The fermentation processes described in the literature to date on the production of biosurfactants include submerged batch and fed-batch fermentations conducted in shaken flasks, in bioreactors or solid-state fermentations (Silva et al., 2021). Therefore, this work aimed to investigate the production of biosurfactant by yeast reducing the production cost using alternative sources of nutrients, as well as to evaluate the potential application of the biomolecule in the formulation of a natural detergent.

2. Material and Methods

2.1 Material

Microorganism

The yeast *Candida guilliermondii* UCP 0992, belonging to the Culture Bank of the Nucleus for Research in Environmental Sciences (NPCIAMB) of the Catholic University of Pernambuco, Brazil. Repeats were performed monthly to maintain cell viability. The strain was used in the tests as a biosurfactant producer.

2.2 Inoculum preparation and biosurfactant production

The inoculum was standardized by transferring the *Candida guilliermondii* UCP 0992 culture to flasks containing 250 mL of Yeast Mold (YMB) medium, with the following composition: yeast extract (0.3%), D-glucose (1%), peptone (0.5%), distilled water q.s.p (100 mL) and incubated under agitation at 200 rpm at 28°C for 48 hours in order to obtain a young culture. After this period, dilutions were performed until a final cell concentration of 5% (v/v) was obtained, subsequently used in the production of the biosurfactant. The production of the biosurfactant was initially carried out by the yeast *Candida guilliermondii* UCP 0992, using the medium described by Konishi et al. (2015), consisting of 50 g of olive oil as a hydrophobic source, 25.0 g of glucose, 1.0 g of yeast extract, 0.5 g of KH2PO4, 0.5 g of MgSO4.7H2O, 0, 3 g of NaNO3 and 1000 mL of distilled water. This medium was modified, replacing glucose with sucrose and olive oil with crude and/or refined cottonseed oil, at the same concentrations described above, varying the pH, as shown in Table 1. In all media, the components were solubilized and sterilized in an autoclave for 20 minutes at 121°C. Initially, the production of the biosurfactant was carried out on a bench scale in 250 mL Erlenmeyer flasks for substrate selection. The fermentations were carried out under orbital agitation at 200 rpm, at a temperature of 30°C for 8 days.

Table 1: Combination of substrate types for biosurfactant production as a function of pH.

Conditions	Substrates	pH			
		3	4	5	6
1	Raw cottonseed oil + glucose	X	X	X	X
2	Refined cotton oil + glucose	X	X	X	X
3	Crude cottonseed oil + sucrose	X	X	X	X
4	Refined cottonseed oil + sucrose	X	X	X	X

2.3. Biosurfactant production in a 5 L bioreactor

Biosurfactant production was carried out in a 5 L bioreactor using the selected medium at 30 ºC under agitation at 350 rpm and aeration at 0.5 vvm for 8 days. After the fermentation period, analyzes were performed to determine biomass (by optical density at 600nm), surface tension, pH (Indicator Paper pH 1–14 Universal indicator) and biosurfactant yield (extraction/isolation).

2.4. Determination of surface tension and Critical Micellar Concentration (CMC) of the biosurfactant

The surface tension and CMC of the biosurfactant were measured in a Sigma 700 tensiometer (KSV Instruments, Finland) using the NUOY ring. Surface tension was measured by immersing the platinum ring in the liquid and recording the force required to pull it across the air-liquid interface.

2.5. Determination of emulsification activity

To determine the emulsification activity, samples of cell-free metabolic fluid were analyzed according to the methodology described by Cooper and Goldenberg (1987): 2.0 mL of an oily substrate (n-hexadecane and frying oil) was added to 2 .0 mL of the metabolic liquid in a graduated tube and the mixture was vortexed for 2 minutes.

The emulsion stability was determined after 24 hours of rest and the emulsification index was calculated by the formula:

$$I_E = \frac{(H_E)}{H_T}\ x\ 100 \quad (1)$$

Where IE is the emulsification index, HE which represents the height of the emulsion and HT which represents the total height of the emulsion

2.6. Evaluation of the stability of the biosurfactant emulsification (effects of pH, addition of NaCl, time under heating and temperature)

The effects of different temperatures (5°C, 70°C, 100°C and 120°C), of different NaCl concentrations (2.0, 4.0, 6.0, 8.0 and 10.0%) and of different pHs (2.0, 4.0, 6.0, 8.0, 10.0 and 12.0) in the activity of the biosurfactant were evaluated in the cell-free metabolic fluid to determine the emulsification activity (Cooper and Goldenberg, 1987) using post-use motor oil as oily substrate. All analyzes were performed in triplicate.

2.7. Biosurfactant isolation and phytotoxicity assessment

The isolation of the *Candida guilliermondii* UCP 0992 biosurfactant was performed according to the methodology described by Daverey and Pakshirajan (2010). The biosurfactant produced was first extracted with ethyl acetate and isopropanol (8:2, v/v) and then the solvent layer containing the biosurfactant was evaporated in a rotary evaporator (Fisatom 804, Brazil) to obtain a quantitative biosurfactant partially purified. The phytotoxicity of the biosurfactant was evaluated at concentrations of ½ CMC, 1x CMC, 2x CMC through seed germination and root growth of cabbage (Brassica oleracea) after five days of incubation in the dark, according to Tiquia et al. (1996), being calculated according to the formulas: Relative seed germination (%) = (number of germinated seeds in the extract / number of germinated seeds in the control) x 100, Relative root length (%) = (mean length root length in extract / mean root length in control) x 100, GI= [(% seed germination) x (% root growth)] / 100 %.

2.8. Preparation of commercial formula of natural detergent and application

The formulation procedure was performed as follows: the biosurfactant was added to 10 ml of distilled water for dissolution. Then, 5mL of sodium hydroxide solution were added while stirring. The system remained at rest for 6 hours for the reaction to complete. Afterwards, it was added to the fatty acid diethanolamide in 10 ml of water, always stirring. Then, sodium chloride and potassium sorbate were added and the volume was completed to 50 mL. The sample was left to rest for 24 hours and then stored in previously sterilized bottles.
For application, a sample of clean white cotton fabric (5 cm^2) was stained with 1 mL of different post-use consumer products (engine oil, soy oil and automotive grease) on the cloth fabric, which was subjected to drying for 12 hours. The stained fabric samples were subjected to comparative analysis by washing with a commercial detergent solution (OMO) with the formulated natural detergent. The stained cloths were placed in separate bottles, one bottle with tap water and commercial detergent (final concentration of 10 mg/mL) and one bottle with tap water and the formulated detergent (final concentration of 10 mg/mL). In all flasks, the final volume was 100 mL. Then, stained tissue samples were maintained under agitation at 200 rpm at room temperature. After the washing time, the tissue samples were removed, washed with water and dried, and all experiments were performed in triplicates. The percentage of stain removal was calculated according to the formulas:

$$R_M = \frac{(P_{TM})}{P_{TL}}\ x\ 100 \quad (2)$$

Where R_M is Stain Removal, P_{TM} represents the post wash and dry stained fabric weight and P_{TL} represents the clean stain free and dry weight of the fabric.

3. Results and discussion

Knowing that the effectiveness of surfactants is determined by the ability to reduce surface tension which is the measure of free energy of the surface per unit area, necessary to bring a molecule from the interior of the liquid to the surface. The reduction of surface and interfacial tensions is considered the main parameter for detecting a surface-active compound in a given medium (Sarubbo et al., 2022).
According to the studies carried out, the media used as a carbon source provided a reduction in surface tension. The surfactants produced in these media were able to reduce surface tension up to 32.18 $mN.m^{-1}$, meaning a 55% reduction in relation to the tension of distilled water (72 $mN.m^{-1}$). Therefore, the reduction in surface tension indicates that *Candida guilliermondii* UCP 0992 managed to degrade the substrates present in the media and

produce the biosurfactant, and can be considered a good producer of biosurfactant. Surface tension data for the biosurfactant produced under the test conditions are shown in Table 2.

Table 2: Surface tension of biosurfactants produced by Candida guilliermondii UCP 0992 in different alternative media

Conditions	Substrates	Superficial tension (mN.m^{-1})			
		pHs			
		3	4	5	6
1	Raw cottonseed oil + glucose	42,97 ± 0,401	41,76 ± 0,368	44,05 ± 0,469	35,24 ± 0,226
2	Refined cotton oil + glucose	45,18 ± 0,444	33,85 ± 0,266	36,70 ± 0,318	40,09 ± 0,375
3	Crude cottonseed oil + sucrose	37,83 ± 0,381	36,11 ± 0190	**32,18 ± 0,117**	39,79 ± 0,240
4	Refined cottonseed oil + sucrose	38,421± 0,298	46,92 ± 0,410	39,79 ± 0,370	34,69 ± 0,011

The surfactants produced by *Candida guilliermondii* UCP 0992 in condition 3 at pH 5, showed a more efficient emulsifying activity on residual frying oil and N-hexadecane, emulsifying around 40 to 78% of these hydrocarbons, respectively, as illustrated in Figure 1.

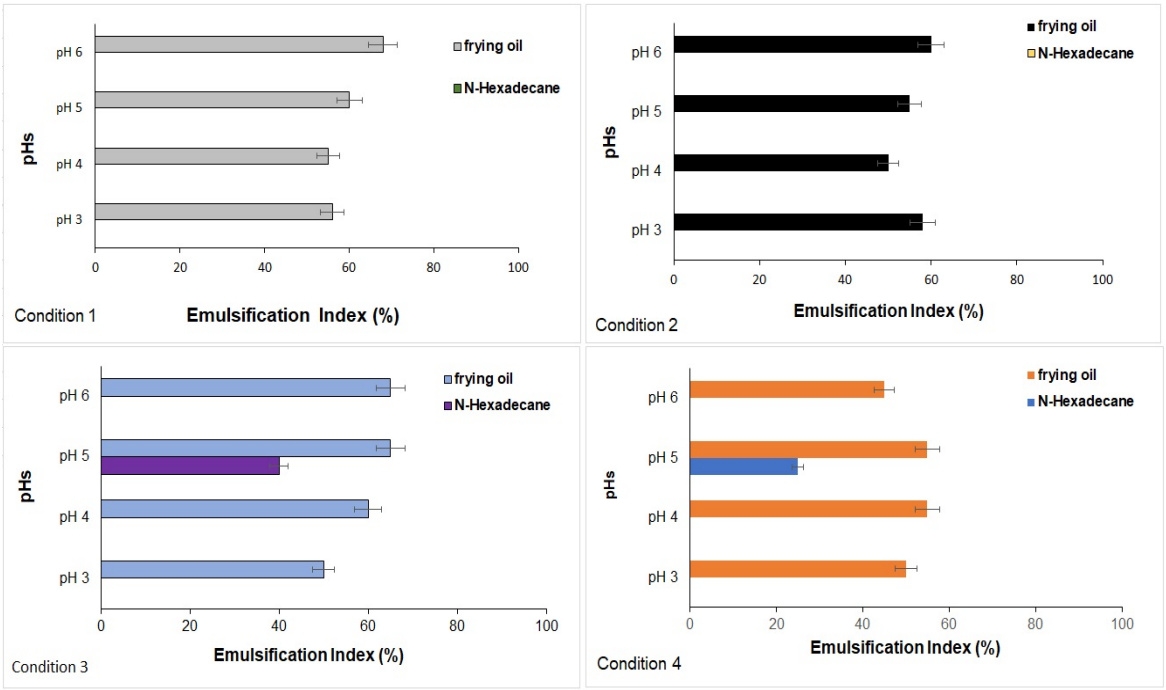

Figure 1: Determination of the Emulsification Index of the biosurfactant produced by Candida guilliermondii UCP 0992 in different alternative media.

From the surface tension and emulsification results obtained for the biosurfactants produced by the yeast *Candida guilliermondii* UCP 0992 under the established conditions, the medium from condition 3, shown in Table 1 at pH 5.0, was selected for production in a 5 L bioreactor, and the Surfactant produced had a surface tension of 32.08 mN.m^{-1}. Yeast cell growth showed an optical density (OD) value of 2.886 nm with a pH of 5.8 at the end of fermentation. The *Candida guilliermondii* UCP 0992 biosurfactant extraction procedure yielded 21 gL^{-1}, with a CMC value of 0.72 gL^{-1}, both using solvents (ethyl acetate and isopropanol).Nowadays, it is understood that the quality of a new surfactant agent is normally evaluated by measurements of surface tension and emulsifying capacity (Farias et al., 2021). In this sense, biosurfactant stability tests were carried out at different values of pH, temperature and increasing NaCl concentrations as a function of emulsifying capacity. The biosurfactants demonstrated stability in their emulsion under all conditions established in the tests (Figure 2).

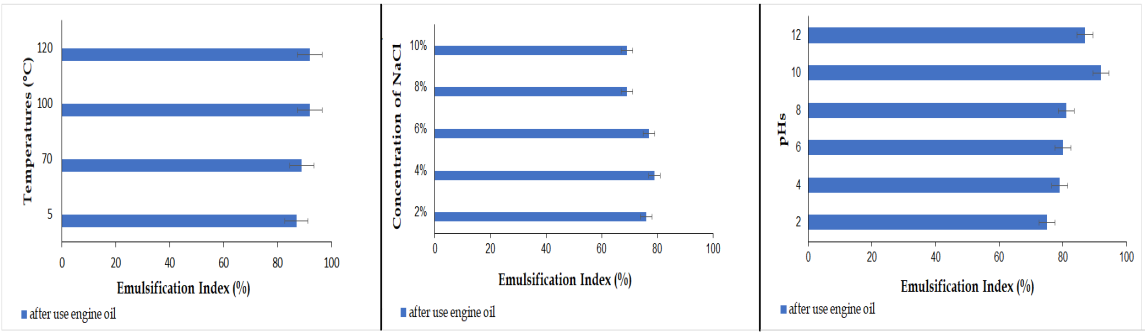

Figure 2: Stability of the Candida guilliermondii UCP 0992 biosurfactant, evaluated under different pH, temperature and NaCl addition conditions through the emulsification index.

The absence of toxicity is of fundamental importance for the application of an ambient product. Eco-toxicity bioassays are analytical methods that allow characterizing the toxicity of chemical substances to be used in domestic and industrial products (detergents) (Almeida et al., 2019). Therefore, the evaluation of the biosurfactant toxicity test was carried out using seeds of the Cabbage-Oxheart (*Brassica oleracea*) and cherry tomato (*Solanum lycopersicum*) vegetables, in order to guarantee that the biosurfactant will not be toxic to the environment. The biosurfactant produced by the yeast, at the concentrations tested (½ CMC, 1x CMC and 2x CMC), showed satisfactory results, since there was a germination index between 86 and 98% in the vegetables used for the tests (Table 3).

Table 3: Phytotoxicity test at different concentrations of biosurfactants produced by Candida guilliermondii UCP 0992.

Germination Index (%)				
Vegetable Seeds	**Biosurfactant Concentration**			
	½ CMC	**CMC**	**2X CMC**	**Água**
Cherry Tomato *(Solanum lycopersicum var. cerasiforme)*	98,66 ± 0,20	94,64 ± 0,30	84,41 ± 0,10	100 ± 0,20
Oxheart Cabbage *(Brassica oleracea)*	92,64 ± 0,10	86,66 ± 0,30	94,4 6± 0,30	100 ± 0,20

Biosurfactants are used in various industrial processes due to their different structures and properties (Rocha e Silva et al., 2020). Preservative compounds are chemical substances whose function is to preserve the initial conditions of the product so that there are no significant changes in its properties over time. The formulation of the natural detergent using the biosurfactants produced by the isolates of *Candida guilliermondii* UCP 0992 showed a very stable behavior with the organoleptic characteristics, pearly white color, pleasant odor, fluid and homogeneous consistency, as well as, it did not cause changes in its surface tension, since they were recorded as 32 mN/m^{-1}. The formulated natural detergent was evaluated in dirt removal tests (soybean oil, post-use engine oil and automotive grease) on cotton fabric, to study the influence of the product (the formulated one) on cleaning, in relation to the removal efficiency. of fabric stains. The results obtained were promising, since the formulated natural detergent showed removal of dirt in 95% for motor oil, 93% for soybean oil and 75% for automotive grease, similar to commercial detergent (OMO), demonstrating that the product remained viable in immediate removal of most contaminants. Thus, the results point to a potential application of the biosurfactant from the strain *Candida guilliermondii* UCP 0992 in the formulation of commercial detergents (Figure 3).

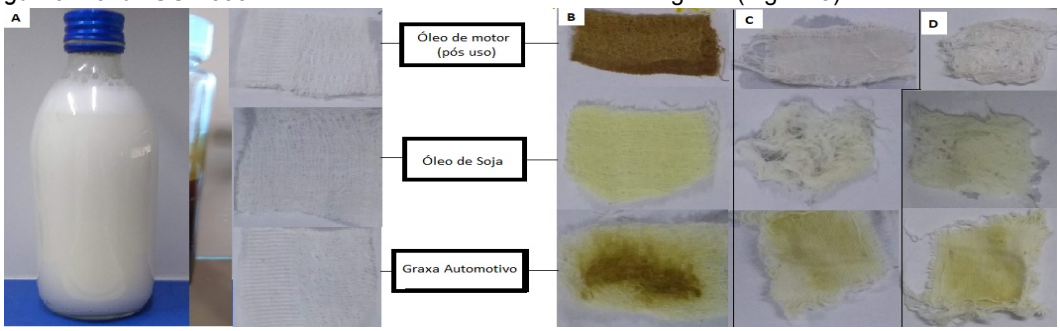

Figure 3: Ilustração da fórmula comercial do detergente natural e o tecido de algodão utilizado (A), bem como, as sujidades no tecido (B), e sua remoção pelo formulado com o biossurfactante de Candida guilliermondii UCP 0992 (C), comparando com o detergente comercial – OMO, pós-lavagem (D).

4.Conclusion

Candida guilliermondii UCP 0992, demonstrated a great biotechnological potential in the production of biosurfactant. In addition, the results of the emulsification, stability, toxicity and formulation experiments clearly demonstrate the viability of applying the biosurfactant produced by yeast as a biotechnological additive for remeasurement processes that consider the preservation and reduction of environmental impacts as essential aspects for maintaining the quality of life and social well-being.

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), EPESA (Centrais Elétricas de Pernambuco S.A.) and Termocabo S.A. This work was also supported by the National Council for Scientific and Technological Development (CNPq), the Foundation for the Support of Science and Technology of the State of Pernambuco (FACEPE) 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 Catholic University of Pernambuco (UNICAP) and to the Advanced Institute of Technology and Innovation (IATI), Brazil.

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