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

Application of a Biosurfactant from *Candida tropicalis* UCP 0996 Produced in Low-Cost Substrates for Hydrophobic Contaminants Removal

Darne G. Almeida [a,b], Rita de Cássia F. Soares da Silva [a,b], Pedro P.F. Brasileiro [b], Juliana M. de Luna [a,b], Maria da Glória C. da Silva [b,c], Raquel D. Rufino [a,b], Andrea F. S. Costa [c], Valdemir A. Santos [a,b], and Leonie A. Sarubbo *[a,b]

[a] Catholic University of Pernambuco, Rua do Príncipe, n. 526, Boa Vista, Cep: 50050-900, Recife-Pernambuco, Brazil
[b] Advanced Institute of Technology and Innovation (IATI), Rua Joaquim de Brito, n. 216, Boa Vista, CEP: 50070-280, Recife, Pernambuco, Brazil
[c] Northeast Biotechnology Network, Federal Rural University of Pernambuco – RENORBIO/UFRPE, Rua Dom Manoel de Medeiros, s/n, Dois Irmãos. CEP: 52171-900, Recife – Pernambuco, Brazil
leonie@unicap.br

Contamination by petroleum and its by-products causes serious damage, which has awakened great attention to the development and application of innovative technologies for the removal of these contaminants. In this sense, biosurfactants have been successfully applied in recent years as remediation agents in impacted environments. Biosurfactants are amphipathic molecules with hydrophobic and hydrophilic portions that act between fluids of different polarities (oil/water and water/oil), allowing access to hydrophobic substrates and causing a reduction in surface tension, an increase in the area of contact of hydrocarbons enhancing mobility, bioavailability and biodegradation of such compounds in the environment. This work describes the environmental application of a biosurfactant produced from *Candida tropicalis* UCP 0996 cultivated in a medium formulated with low-cost substrates. Fermentations were conducted in the medium supplemented with 2.5 % waste frying oil, 2.5 % corn steep liquor and 2.5 % molasses at 28 ºC during 120 h under 200 rpm. The medium surface tension was reduced from 55 mN/m to 30.4 mN/m. A Critical Micelle Concentration (CMC) of 0.6 % was obtained from the isolated biosurfactant. Biosurfactant application demonstrated an ability to disperse 70.95 % of motor oil on seawater surface at 5xCMC. Stone washing tests showed 46.02 and 56.18 % removal at 2xCMC and 5xCMC of biosurfactant concentration, respectively, and testing for oil biodegradation showed results around 73.8 % for motor oil biodegradation by indigenous marine bacteria and fungi from the Suape Petrochemical Complex seawater, Pernambuco State, Brazil. The results obtained with the biosurfactant produced by *Candida tropicalis* UCP 0996 under the conditions tested above show the promising properties of this biomolecule for use in bioremediation of hydrophobic compounds in marine environment.

1. Introduction

The release of petroleum and petroleum byproducts into the environment is one of the main causes of global pollution and has become a focus of great concern both in industrialized and developing countries once oil pollution can have dramatic detrimental effects to the environment and cause significant damages to resident organisms (Almeida et al., 2017a). The need for remediating polluted areas has paved the way for development of new technologies to detoxify contaminants not only through chemical or physical methods, but through biological techniques as well.

Bioremediation is a set of technologies that make the removal of contaminants possible, or failing that, make a number of contaminants less harmful by means of biological activity (Silva et al., 2014). For the success of bioremediation technologies, microorganisms employment with the appropriate metabolic abilities for

biodegradation and able to transform contaminants into less toxic substances is the most important requisite on oil spill bioremediation (Al-Wasify and Hamed, 2014; Cerqueira et al., 2012).
Biosurfactants have been successfully applied in recent years as remediation agents in aquatic environments. These compounds are amphipathic molecules with hydrophobic and hydrophilic portions that act between fluids of different polarities (oil/water and water/oil), allowing access to hydrophobic substrates and causing a reduction in surface tension, an increase in the area of contact of hydrocarbons enhancing mobility, bioavailability, and biodegradation of such compounds (Silva et al., 2014).
Regarding their ability to solubilize hydrocarbons by partitioning them into the surfactant micelles above the CMC, biosurfactants are used in different remediation processes originated from the oil industry. One of the main obstacles to the expansion of the biosurfactant market for in situ remediation is the lack of knowledge about its effects on the environment and the toxicity of these substances (Almeida et al., 2016).
The *Candida tropicalis* yeast has been widely studied by several researchers as a hydrocarbons degrading potent agent (Almeida et al., 2017b; Chandran and Das, 2011; Farag and Soliman, 2011). Further recent studies have reported that this species has also the metabolic capacity to produce biosurfactant when cultivated on water-immiscible substrates (Batista et al., 2010; Chandran and Das, 2012; Verma et al., 2015).
Therefore, in the present study, the biosurfactant produced from *Candida tropicalis* UCP 0996 was evaluated as a dispersing agent in the spilled oil and a biodegradation improver in bioremediation processes.

2. Materials and Methods

2.1 Materials

All chemicals were reagent grade. Canola waste frying oil was obtained from a local restaurant in the city of Recife, state of Pernambuco, Brazil. Cane molasses was obtained from the municipality of Victória de Santo Antão, PE, Brazil. Corn steep liquor was obtained from Corn Products do Brasil in the municipality of Cabo de Santo Agostinho, state of Pernambuco, Brazil. Growth media were purchased from Difco Laboratories (USA).

2.2 Yeast strain and preparation of inoculum

A *Candida tropicalis* UCP0996 strain was provided from the culture collection of the Catholic University of Pernambuco, Recife city, Pernambuco, Brazil. Microorganism was maintained at 5 °C on yeast mold agar 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. Inoculum was prepared by transferring cells grown on a slant to 250- mL Erlenmeyer flasks containing 50 mL of yeast mold broth (YMB). YMA and YMB media initial pH was adjusted to 5.5. Seed culture cultivation conditions were 28 °C, 200 rpm and incubation 24 h.

2.3 Biosurfactant production

The production of biosurfactant was performed in a basal medium composed of 2.5 % cane molasses, 2.5 % waste frying oil and 2.5 % corn steep liquor. Initial pH was adjusted to 5.5. 500-mL shake flasks were kept under 200 rpm orbital agitation for 120 h at 28 °C. After fermentation 120 hours, surface tension and biosurfactant yield were determined (Rocha e Silva et al., 2013).

2.4 Surface tension measurement

Surface tension was determined in the cell-free broth obtained by centrifuging the cultures at 10,000 × g for 15 min. Surface tension was determined with a Tensiometer (Sigma 700, KSV Instruments Ltd., Finland), using the Du Nouy ring method at room temperature (Silva et al., 2014).

2.5 Isolation of biosurfactant

After cultivation, biosurfactant was recovered from the cell-free broth by cold acetone precipitation and isolated biosurfactant concentration was expressed in g/L, according to Santos et al. (2016).

2.6 Critical micelle concentration

The critical micelle concentration (CMC) was determined by measuring the surface tension of the dilutions of the isolated biosurfactant in distilled water up to a constant surface tension value. 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 was obtained by plotting surface tension against surfactant concentration and expressed as (%) of biosurfactant.

2.7 Application of the biosurfactant in hydrophobic contaminant spreading

Oil displacement test was carried out slowly by dropping of 15 µl of motor oil onto the surface of 40 ml of seawater layer contained in a Petri dish (15 cm in diameter) that spread all over the water surface area. This was followed with the addition of 10 µl of the formulated biosurfactant and aqueous solutions containing the isolated surfactant at 1xCMC, at 2xCMC and 5xCMC onto the surface of the oil layer. The average value of the diameters of the clear zones of triplicate experiments was measured and recorded then calculated as percentage of the Petri dish diameter (Ohno et al., 1993).

2.8 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 formulated biosurfactant and with the isolated biosurfactant at 1xCMC, at 2xCMC and 5xCMC concentration. After the culture process, the percentage of removal through washing was calculated. Following the washing of the porous surface, the samples were treated with 50 mL of hexane twice for the removal of residual oil. The solvent was rotoevaporated at 50 ºC and the amount of oil removed was determined by gravimetry (Sarubbo et al., 2012).

2.9 Bioremediation study

Bioremediation tests were performed according to the method mentioned in the Standard Methods for the Examination of Water and Wasterwater. In brief, 250 mL Erlenmeyer flasks were filled with 100 mL fresh seawater obtained from the Suape Petrochemical Complex, Pernambuco State, Brazil, 1.0 % of motor oil and solutions with isolated-biosurfactant concentrations 1xCMC, at 2xCMC and 5xCMC. The flasks were incubated at 28 °C on an orbital shaker rotating at 150 rpm. Shake flasks were withdrawn after 1, 7,14, 21 and 28 days of incubation and then analyzed for the number of microorganisms using the most probable number (MPN) (Rocha e Silva et al., 2013).

3. Results and Discussion

3.1 Biosurfactant production

Maximum biosurfactant production was 4.11 g/L and occurred in the stationary growth phase, after 120 h of cultivation. Surface tension of the medium was reduced from 55 mN/m to 30.4 mN/m. After the extraction and isolation process, isolated biosurfactant concentrations versus the surface tensions were plotted in Figure 1. As can be seen, the water surface tension decreased gradually with increasing isolated biosurfactant concentration from 70 to 25.6 mN/m with a biosurfactant concentration of 0.06 %, and then remained constant. Further increase in the biosurfactant concentration did not reduce the surface tension of water, indicating that the CMC was reached at this concentration. The CMC of the *C. tropicalis* biosurfactant is within the CMC values reported by the literature for different types of biosurfactants produced by other *Candida* species, as the biosurfactants from *C. lipolytica* UCP 0988 (0.03 %) (Rufino et al., 2014), from *C. glabrata* (2.5 %) (Luna et al., 2009), from *C. antarctica* (0.6%) (Adamczak and Bednarski, 2000) and from *C. sphaerica* UCP 0995 (0.025%) (Luna et al., 2013).

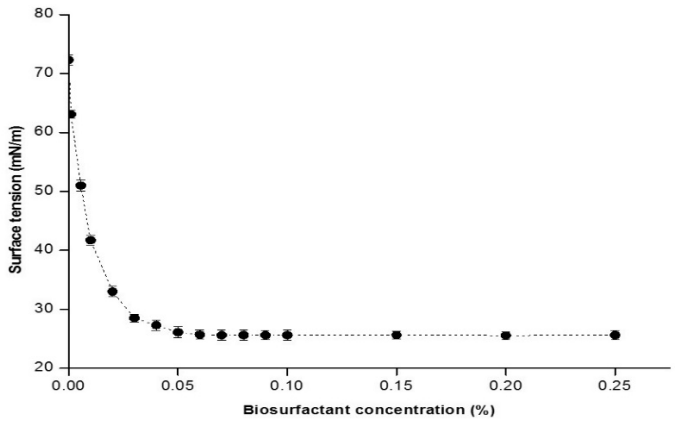

Figure 1: Surface tension versus concentration of the isolated biosurfactant from C.tropicalis UCP 0996

3.2 Application of the biosurfactant in hydrophobic contaminant spreading

Many processes carried out by the oil industry contaminate the marine environment. Eventually, a part of the process, oil accidentally reaches the seawater and, in turn, surfactants must be used together with other containment measures. In this study, it was evaluated the dispersing ability of the formulated biosurfactant from *C. tropicalis* (Table 1).

As can be seen, the best dispersion indices found were 70.95 % for the isolated. Luna et al. (2016) also evaluated the dispersion capacity of the biosurfactant produced by *C. bombicola*. As a result, the isolated biosurfactant showed a maximum dispersion rate 50 % of motor oil.

Table 1: Motor oil dispersion by biosurfactant from C. tropicalis UCP 0996 cultivated in distilled water supplemented with 2.5 % waste frying oil, 2.5 % corn steep liquor and 2.5 % cane molasses

Removal agent	Dispersion (%)
1 x CMC	45.23 ± 0.11
2 x CMC	53.80 ± 0.12
5 x CMC	70.95 ± 0.3

3.3 Washing of hydrophobic compound adsorbed to porous surface

Few methods are suitable for cleaning contaminants in coral reefs, which are very delicate and difficult to access. However, the use of dispersants is an attractive method when an ecosystem is exposed to an oil spill (Sobrinho et al., 2013). The literature has little to offer about oil removal on porous surfaces. However, Luna et al. (2016) evaluated the removal capacity of the biosurfactant from *C. bombicola* showing a removal of 70 % of the motor oil adsorbed on a porous surface. In the present study, a removal of about 66.18 % was found to be the best result for the isolated biosurfactant from *C. tropicalis* (Table 2), showing the feasibility of its application as a biological dispersant to remove hydrophobic pollutants in sensitive ecosystems such as coral reefs.

Table 2: Removal of motor oil adsorbed on marine stones by the formulated and isolated biosurfactant produced by C. tropicalis UCP 0996

Removal agent	Removal (%)
1 x CMC	42.01 ± 0.12
2 x CMC	56.02± 0.21
5 x CMC	66.18± 0.4
Control (seawater)	2.35± 0.1

3.4 Bioremediation study

Biosurfactant effect on the motor oil biodegradation through the activity of indigenous marine bacteria and fungi was evaluated during 28 days (Figure 2). As can be seen, the results related to the addition of biosurfactants were superior to the control (biosurfactant absence) as predicted since the isolated biosurfactant from *C. tropicalis* had the ability to stimulate growth of indigenous microorganisms and a consequent motor oil biodegradation. Under all conditions tested, bacterial growth reached its maximum on the fourteenth day, decreasing microbial cells number after this period. As for fungi, there was a marked increase, starting from the fourteenth day, which may be related to lower competition as a result of the decline in the number of bacteria from this period. At the concentrations of 1xCMC, biosurfactant from *C. tropicalis* had already stimulated almost 60% biodegradation, while at 3xCMC and 5xCMC, the oil biodegradation was above 70%, with maximum of 73.8 %. This demonstrates that the biosurfactant already presents an excellent activity in low concentrations, which represents an advantage for the industrial production of this biomolecule as a bioremediation agent applied in oil spills. Similar results were obtained by Santos et al. (2016).

They observed that the presence of the biosurfactant from *C. lipolytica* favored the growth of indigenous microorganisms in seawater at the concentrations of ½xCMC, CMC and 3×CMC throughout 30 days of cultivation. Rocha e Silva et al. (2013) also obtained an increase on the indigenous microorganisms growth throughout 30 days of cultivation in the presence of the biosurfactant from *P.cepacia*.

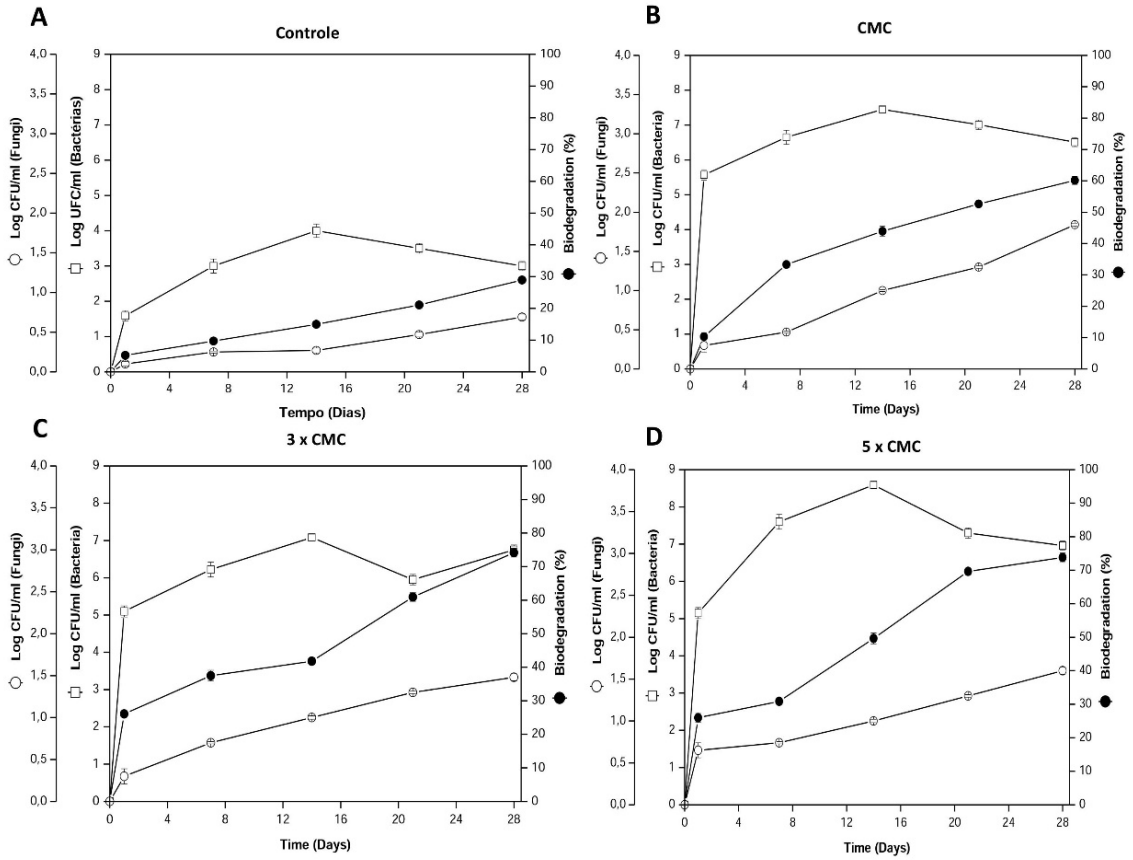

Figure 2: Influence of biosurfactant from C.tropicalis UCP 0996 on growth of bacteria and fungi indigenous in seawater. Microbial growth in the (A) biosurfactant absence (B) biosurfactant at 1xCMC (C) at 3xCMC (D) at 5xCMC

4. Conclusions

The biosurfactant produced by *C. tropicalis* cultivated in a low-cost medium demonstrated a great potential of application as an oil spill remediation agent in marine environments. The biosurfactant showed presented a good surface tension reduction and great biosurfactant yield, and was able to remove oil from sea stones and cause an excellent dispersion of oil stains in seawater, making this biomolecule effective for application in the bioremediation processes as a commercial stable dispersant.

Acknowledgments

This study was funded by the Research and Development Program from National Agency of Electrical Energy (ANEEL), the Candeias Energy Company (CEC) from Global Group, through the Project code PD-06961-0005/2016, 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.

Reference

Adamczak, M., Bednarski, W., 2000, Influence of medium composition and aeration on the synthesis of biosurfactants produced by *Candida antarctica*. Biotechnol. Lett. 22, 313–316.

Almeida, D. G., Silva, M. G. C., Barbosa, R. N., Silva, D. S. P., Silva, R. O., Lima, G. M.S., Gusmão, N. B., Sousa, M. F. V. Q., 2017a, Biodegradation of marine fuel MF-380 by microbial consortium isolated from seawater near the petrochemical Suape Port, Brazil. Int. Biodeterior. Biodegradation.116, 73–82.

Almeida, D. G., Soares da Silva, R. C. F., Luna, J. M., Rufino, R. D., Santos, V. A., Banat, I. M., Sarubbo, L. A., 2016, Biosurfactants: Promising Molecules for Petroleum Biotechnology Advances. Front Microbiol. 7, 1–14.

Almeida, D. G., Soares da Silva, R. F. C., Luna, J. M., Rufino, R. D., Santos, V. A., Sarubbo, L. A., 2017b, Response Surface Methodology for Optimizing the Production of Biosurfactant by *Candida tropicalis* on Industrial Waste Substrates, Artic. Front. Microbiol. 8. doi:10.3389/fmicb.2017.00157.

Al-Wasify, R. S., Hamed, S. R., 2014, Bacterial biodegradation of crude oil using local isolates. Int. J. Microbiol. 2014, 1–8.

Batista, R. M., Rufino, R. D., Luna, J. M., Souza, J. E. G., Sarubbo, L. A., 2010, Effect of medium components on the production of a biosurfactant from *Candida tropicalis* applied to the removal of hydrophobic contaminants in soil, Water Environ. Res. 82, 418–425.

Cerqueira, V. S., Hollenbach, E. B., Maboni, F., Camargo, F. A. O., Peralba, M. C. R., Bento, F. M., 2012, Bioprospection and selection of bacteria isolated from environments contaminated with petrochemical residues for application in bioremediation, World J. Microbiol Biotechnol. 28, 1203–1222.

Chandran, P., Das, N., 2011, Degradation of diesel oil by immobilized *Candida tropicalis* and biofilm formed on gravels. Biodegradation. 22, 1181–1189, doi:10.1007/s10532-011-9473-1.

Farag, S., Soliman, N. A., 2011, Biodegradation of crude petroleum oil and environmental pollutants by *Candida tropicalis* strain. Braz. Arch. Biol. Technol . 54, 821-830. doi: 10.1590/S1516-89132011000400023

Luna J. M., Sarubbo L. A., Campos-Takaki G. M. A., 2009, New biosurfactant produced by *Candida glabrata* UCP1002: characteristics of stability and application in oil recovery, Braz. Arch. Biol. Technol. 52, 785.

Luna, J. M., Rufino, R. D., Sarubbo, L. A., Campos-Takaki, G.M., 2013, Characterisation, surface properties and biological activity of a biosurfactant produced from industrial waste by *Candida sphaerica* UCP0995 for application in the petroleum industry. Colloids and Surfaces B: Biointerfaces. vol.102, p.202–209.

Luna, J. M., Santos Filho, A. S., Rufino, R. D., Sarubbo, L. A., 2016, Production of Biosurfactant from *Candida bombicola* URM 3718 for Environmental Applications, Chem. Eng. Trans. 49, 583–588. doi:10.3303/CET1649098.

Ohno, A., Takashi, A., Shoda, N., 1993, Production of antifungal peptide antibiotics iturin by *Bacillus subtilis* NB22 in solid state fermentation, J. Biosci. Bioeng. 75, 23–27.

Rocha e Silva, N. M. P., Rufino, R. D., Luna, J. M., Santos, V. A., Sarubbo, L. A., 2013, Screening of *Pseudomonas* species for biosurfactant production using low-cost substrates. Biocatal. Agric. Biotechnol. 3, 132–139.

Rufino, R. D., Luna, J. M., Campos-Takaki, G. M., Sarubbo, L. A., 2014, Characterization and properties of the biosurfactant produced by *Candida lipolytica* UCP 0988, Electron. J. Biotechnol. 17, 34–38.

Santos, D. K. F., Rufino, R. D., Luna, J. M., Santos, V. A., Sarubbo, L. A. 2016, Biosurfactants: Multifunctional biomolecules of the 21st century, Int. J. Mol. Sci. 17, 1–31, doi:10.3390/ijms17030401.

Sarubbo, L. A., Rufino, R. D., Luna, J. M., Farias, C. B. B., Santos, V. A., 2012, Production of biosurfactants for application in the removal of hydrophobic contaminants originated by the petroleum industry. Chem. Eng. Trans. 27, 7–12.

Silva, R. C. F. S., Almeida, D. G., Rufino, R. D., Luna, J. M., Santos, V. A., Sarubbo, L. A., 2014, Applications of biosurfactants in the petroleum industry and the remediation of oil spills, Int. J. Mol. Sci. 15, 12523–12542. doi:10.3390/ijms150712523.

Sobrinho, H. B. S., Luna, J. M., Rufino, R. D., Porto, A. L. F., Sarubbo, L. A., 2013, Application of biosurfactant from *Candida sphaerica* UCP 0995 in removal of petroleum derivative from soil and sea water. J. Life Sci. 7, 559–569.

Verma, A., Gupta, N., Verma, S. K.; Das, M. D. J., 2015, Multifactorial approach to biosurfactant production by adaptive strain *Candida tropicalis* MTCC 230 in the Presence of Hydrocarbons. J. Surfactants Deterg. 18, 145–153. doi:10.1007/s11743-014-1608-z