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Bioemulsifier produced by *Yarrowia lipolytica* using residual glycerol as a carbon source

Joselma Ferreira da Silva^a, Lucas Albuquerque Rosendo da Silva^b, Marta Ribeiro Barbosa^a, Laureen Michelle Houllou^a, Carolina Barbosa Malafaia^a

^a Centro de Tecnologias Estratégicas do Nordeste-CETENE, Av. Prof. Luís Freire, n. 01, Cidade Universitária, Recife-PE, Brasil. CEP: 50.740-540. Corresponding author: joselma.fsilva@gmail.com.

^b Universidade Católica de Pernambuco, Rua do Príncipe, n. 526, Boa Vista, Recife-PE, Brasil. CEP: 50.050-900.

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ABSTRACT

Bioemulsifier is bioactive molecules produced by different microorganisms with reducing power and surface and interfacial tension. Among the microorganisms producing this molecule is yeast, which can produce different bioemulsifiers in different substrates. Undoubtedly, this biomolecule has excellent potential for industrial applications, but high production costs are the biggest problem in production. Aiming at cost reduction the present study using crude residual glycerol for biosurfactant production by *Yarrowia lipolytica*. Then isolates were grown in residual glycerol compound medium, rotating 200 rpm at 28°C for 48 hours. Bioemulsifier production was observed by analysis of dry biomass, pH, surface tension and emulsification index. The results indicated that the emulsion produced from biosurfactant using glycerol as a carbon source by *Y. lipolytica* has the potential for bioemulsifier production. All isolates obtained similar results for all analyzes, indicating that this species has a linear production among the isolates. Biomass reached $10.08 \pm 0.62 \text{ g.L}^{-1}$, there was a sharp drop in pH reaching 4.6, surface tension averaged 41.7 mN.m^{-1} and emulsification index reached 56%. The isolates tested show potential for bioemulsifier production using glycerol as an unconventional carbon source.

Keywords: Yeast, industrial waste, emulsion, surface tension.

Introduction

Biosurfactants are amphiphilic molecules which have a hydrophilic moiety composed of amino acids, peptides, esters, carbohydrates or hydroxyl phosphate, alcohol, and carboxyl groups; and a hydrophobic portion of long-chain fatty acid jams, fatty acid β -hydroxy alkyl (Rufino et al., 2014). They are surfactants, that is, with the potential to decrease the surface and interfacial tension between phases (gas, liquid, and solid) and to stabilize solutions by the formation of amphipathic microemulsions (Campos et al., 2013; Singh, Hamme & Ward, 2007). It is produced in hydrocarbon fermentation processes by microorganisms such as bacteria, yeast, and filamentous fungi and is excreted in the culture medium (Banat et al., 2010; Santos et al., 2016; Singh, 2012).

Biosurfactants are considered less environmentally impacting molecules, even though they have the same properties as a commercial surfactant. However, due to their

structural versatility, they have advantages over synthetics (Khan et al., 2017; Gudiña et al., 2013), as higher biodegradability and lower toxicity are also useful in extreme temperatures, solubilization, emulsification, higher formation of foam, high selectivity, pH and salinity (Csutak, Stoica & Vassu, 2012; Lawniczak, Marecik & Chrzanowski, 2013). These have been successfully applied in recent years as bioremediation agents in soils and aquatic environments (Sarubbo, Luna & Rufino, 2015).

Biosurfactants production is described in some microorganisms; among them *Yarrowia lipolytica* yeast (Almeida et al., 2016) stands out as a potential industrial value and is easily found in nature (Almeida et al., 2016; Santos et al., 2016). This yeast produces a highly efficient emulsifier important in the hydrophobic process of substrate assimilation (Beopoulos et al., 2010).

One of the major concerns for biosurfactant production is the high cost, which makes these substances not economically

competitive with synthetic surfactants (Thavasi, Jayalakshmi & Banat, 2011). As a strategy to reduce the high costs of biosurfactant production, processes have been developed for biosurfactant production using agro-industrial waste as a carbon source. These wastes have high pollutant potential but are not considered waste because it has an added value and can be reused in other processes. It minimizes the environmental impact of this waste by improper disposal (Amaral et al., 2006; Laufenberg, Kunz & Nystroem, 2003). Alternative carbon sources such as vegetable oil waste and industrial waste have been evaluated as a substrate for the production of biosurfactants, coffee processing waste, fruit, cassava, corn, soybean, glycerol, among others (Saharan, Sahu & Sharma, 2011). Among these alternatives, crude residual glycerol, a byproduct of the biodiesel production process, has been highlighted as an essential nutrient-rich substrate as a cheap carbon source (Silva et al., 2014).

The objective of the study was to investigate biosurfactant production and its surfactant potential by isolates of *Yarrowia lipolytica* using crude residual glycerol as an alternative carbon source.

Material and Methods

Microorganism

Yarrowia lipolytica isolates (CTN-08, CTN-10, CTN-14, CTN-90, and CTN-136) were obtained by direct isolation of dairy from the wild region of Pernambuco. Yeasts were isolated by depletion in Petri dishes containing YPDA medium (yeast extract - 10 g.L⁻¹, peptone - 20 g.L⁻¹, dextrose - 20 g.L⁻¹, agar - 20 g.L⁻¹) with chloramphenicol and amoxicillin antibiotic. were identified by protein profile using the Matrix Associated Laser Desorption-Ionization Time of Flight (MALDI-TOF - Bruker) technique. After isolation, the strains were kept in YPDA medium at 28°C for 24 h.

Inoculum preparation

The initial inoculum was prepared in 500 ml Erlenmeyer flasks containing 200 mL of YSGB broth (yeast extract - 5g.L⁻¹, ammonium sulfate - 10 g.L⁻¹, crude residual glycerol - 30 g.L⁻¹). They were inoculated with generous *Y. lipolytica*, then incubated at 28°C at 200 rpm for 48h. The inoculum constituted 10% of the final culture volume standardized by optical density (OD) between 0.8 to 0.9 to 600nm.

Fermentation

Fermentation was performed using the same medium as above and incubated under the

same conditions. The evaluation of microbial growth through dry biomass, pH, determination of total reducing sugars, emulsification index, and emulsifier production were done. Samples of 10 mL of the fermented were collected every 24 h from time 0 h to 48 h.

Cell growth determination

For biomass determination, 5 mL of the fermentate was centrifuged at 5000 x g for 20 min. Then the supernatant was transferred to a new clean tube for further analysis. The biomass was washed twice with distilled water and centrifuged again under the same conditions. Then water was discarded, and the cell pellet was oven-dried at 60°C for 24h. Biomass was determined by weighing on an analytical balance.

Determination and pH

The pH was determined using a digital potentiometer (pH 1800 PG, Gehaka) at room temperature 25°C using 3 mL of the cell-free fermented broth.

Superficial tension

The method used was described by Du Nouy (1925) with the tensiometer automatic model (Sigma 700 KSV Instruments LTD, Finlândia) at room temperature. Surface tensions were performed in the cell-free fermented broth. The tests were performed in triplicate.

Extraction and yield of bioemulsifier

Cell-free broth (25 mL) was acidified to pH 2 using 6 M HCl and maintained overnight (Bharali et al., 2011). Then, the sample was transferred to the separatory funnel, and 50 mL chloroform and 50 mL methanol was added every 10 minutes until the final solvent volume of 200 mL was completed and manually homogenized. Bioemulsifier was obtained by centrifugation at 400 x g for 15 min at 24°C. The precipitate was resuspended in 5 mL of lyophilized distilled water (Silva et al., 2014).

Emulsification Index

The emulsification index test was made according to Cirigliano & Carman (1984). The samples were subjected to E24 emulsification index analyzes into a test tube 2.0 mL of sample (previously thawed at room temperature) was added, and 2.0 mL of mineral oil was added and then vortexed for 2 min. After 24 h at rest, the height (mm) of the formed water/oil emulsion and the total height were read. Emulsification index (E₂₄ - %) was defined as the centesimal

relationship between emulsion layer height and total height, according to Equation 1.

$$E_{24 (samples)} = \frac{A_{emulsion}}{A_{total}} * 100 \quad \text{Eq.(1)}$$

Statistical Analysis

Microbiological tests were performed, at least in biological triplicate. The chemical tests were performed in triplicate. The results were expressed as mean \pm standard deviation. Tukey test evaluates the differences between groups, and

the values were considered statistically significant if compared to the significance level of $p < 0.05$.

Results and Discussion

Cell growth (Figure 1) in the proposed culture medium using crude glycerol as a carbon source showed a similar profile for all *Y. lipolytica* isolates tested. Exponential growth was observed during the culture time, with CTN-90 isolate showing the highest cell density, reaching $10.08 \pm 0.62 \text{ mg.L}^{-1}$. The significant increase in biomass indicates that the yeast was able to consume glycerol, indicating that its use may be appropriate.

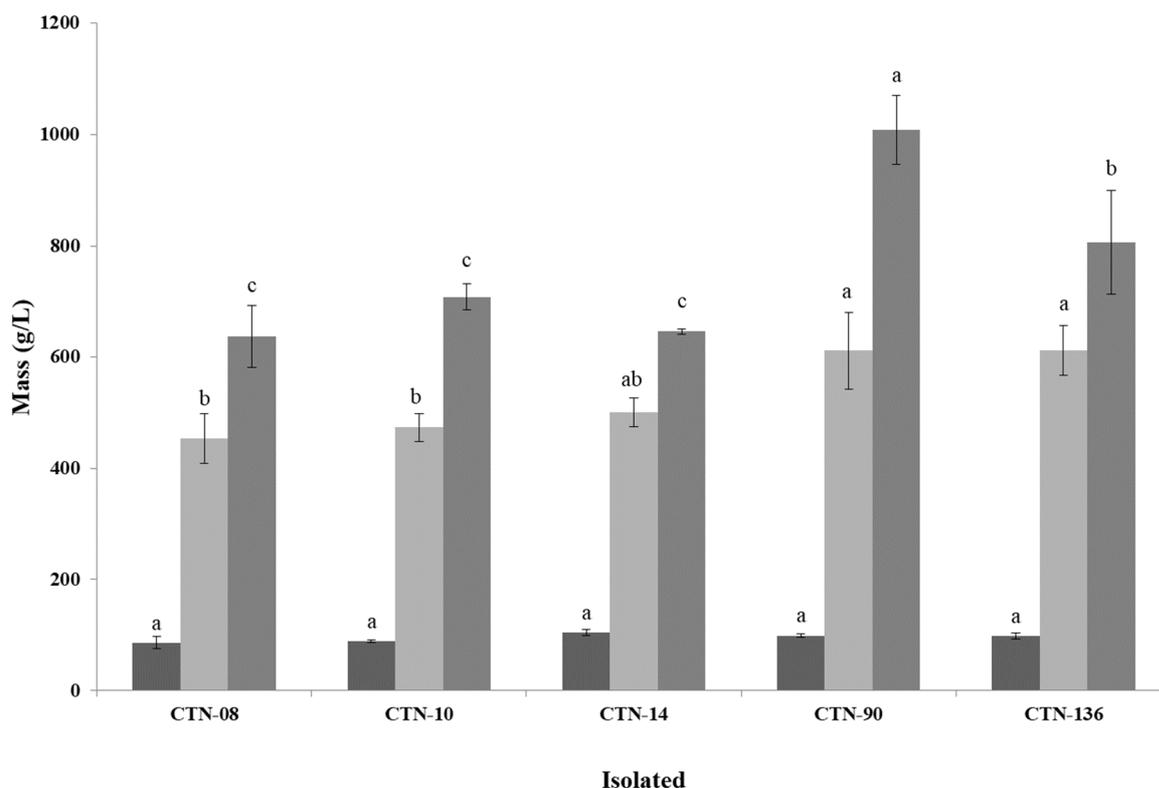


Figure 1. Biomass obtained over time (■ - 0h, ■ - 24h, and ■ - 48h) by different *Yarrowia lipolytica* isolates, cultivated with residual glycerol as carbon source ($p < 0.05$).

The composition of the culture medium has a fundamental role in the type and concentration in the production of biosurfactants, so the choice of a suitable carbon source for cultivation is of fundamental importance (Silva et al., 2010). High carbohydrate or lipid industrial waste is ideal for use as a substrate (Banat et al., 2010) mainly because of its low cost.

Crude residual glycerol is a residue of biodiesel industries and becomes an alternative carbon source for the production of various secondary metabolites, including biosurfactant (Fontes et al., 2012; Souza et al., 2017). Lima et al. (2013) performed an assay for biosurfactant production using *Y. lipolytica* with crude glycerol

(30 g.L^{-1}), and at the end of the process, the microorganisms showed maximum cell growth of 5.9 mg.L^{-1} in 48 h, about half of what was observed in this study. In this sense, the isolates appear to have been more efficient in using this substrate for cell multiplication.

During cultivation, the pH was monitored over time (Figure 2), and it was possible to observe acidification of the medium. Initially, the pH was more neutral, around 7.6, in the first 24h, there was a sharp drop in pH reaching approximately 5.6, and at 48h reached 4.6. This behavior was uniformly observed among the *Y. lipolytica* isolates tested, and no statistical distinction was observed between them.

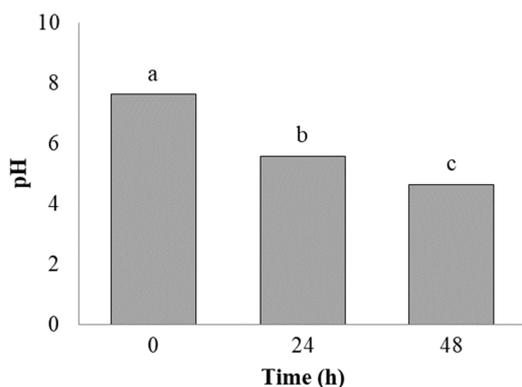


Figure 2. pH values throughout the culture of *Yarrowia lipolytica* isolates, cultivated with residual glycerol as a carbon source ($p < 0.05$).

Among the *Y. lipolytica* isolates tested, and no statistical distinction was observed between them. *Yarrowia lipolytica* is capable of producing and excreting in the medium a wide range of organic acids, such as tricarboxylic acid, isocytic acid, 2-ketoglutaric acid, and pyruvic acids, from low-cost carbon sources, promoting the reduction of carbon dioxide. The pH of the medium is an essential indicator of biosurfactant production by microorganisms (Fickers et al., 2005). Bednarski et al. (2004) state that the constant acidity found in the culture medium is a parameter that correlates the efficacy of glycolipid synthesis by yeasts, such as *Candida antarctica* and *C. apicola*.

Surface tension is one of the factors that suggest the production of yeast bioemulsifying molecules. The reduction in surface tension observed in the cell-free fermented broth obtained from the five *Y. lipolytica* isolates tested can be observed (Table 1). Results indicate a high emulsifying power, on average of 41.7 mN.m^{-1} . The results indicated that there was no significant difference in the emulsifying power produced between the isolates. These data indicate the ability of this species to produce bioemulsifier in the culture medium from crude residual glycerol.

Table 1. Surface tension values obtained from the different isolates of *Yarrowia lipolytica*.

Isolated	Superficial tension
CTN-08	45.0 ± 0.6
CTN-10	38.7 ± 4.0
CTN-14	42.5 ± 1.7
CTN-90	40.9 ± 1.8
CTN-136	41.3 ± 1.4

Superficial reduction after 24 h was also observed by Rufino et al. (2014), studying biosurfactant production by *Y. lipolytica* verified the reduction of tension when they were in exponential phase, i.e., during cell growth. Haba et al. (2000) found a reduction in surface tension in media from 57 mN.m^{-1} to values ranging from 35 mN.m^{-1} to 40 mN.m^{-1} , using low-cost substrates (oil used for frying foods) by different yeast species. Surface tension was reduced to around 43 mN.m^{-1} using *Y. lipolytica* fermented broth, similar data was also described by (Souza et al., 2017). This reduction was being reached at 72 h, using *Wickerhamomyces anomalus* as a fermenting organism from residual glycerol as a carbon source.

The emulsification index (Figure 3) determines the ability of a molecule to emulsify hydrocarbons, and to be considered an efficient emulsifier, the molecule must be able to maintain at least 50% of the emulsification after 24h of rest. In the fermented liquids tested it was observed that on average there was the maintenance in about 56%. This value was observed in the fermented both 24h and 48h of cultivation. Statistically, there was no significant difference when comparing cultivation times and also when comparing the different isolates with each other. It is suggested that increased production of bioemulsifiers in the medium allows for increased hydrocarbon solubilization to be used to facilitate yeast growth.

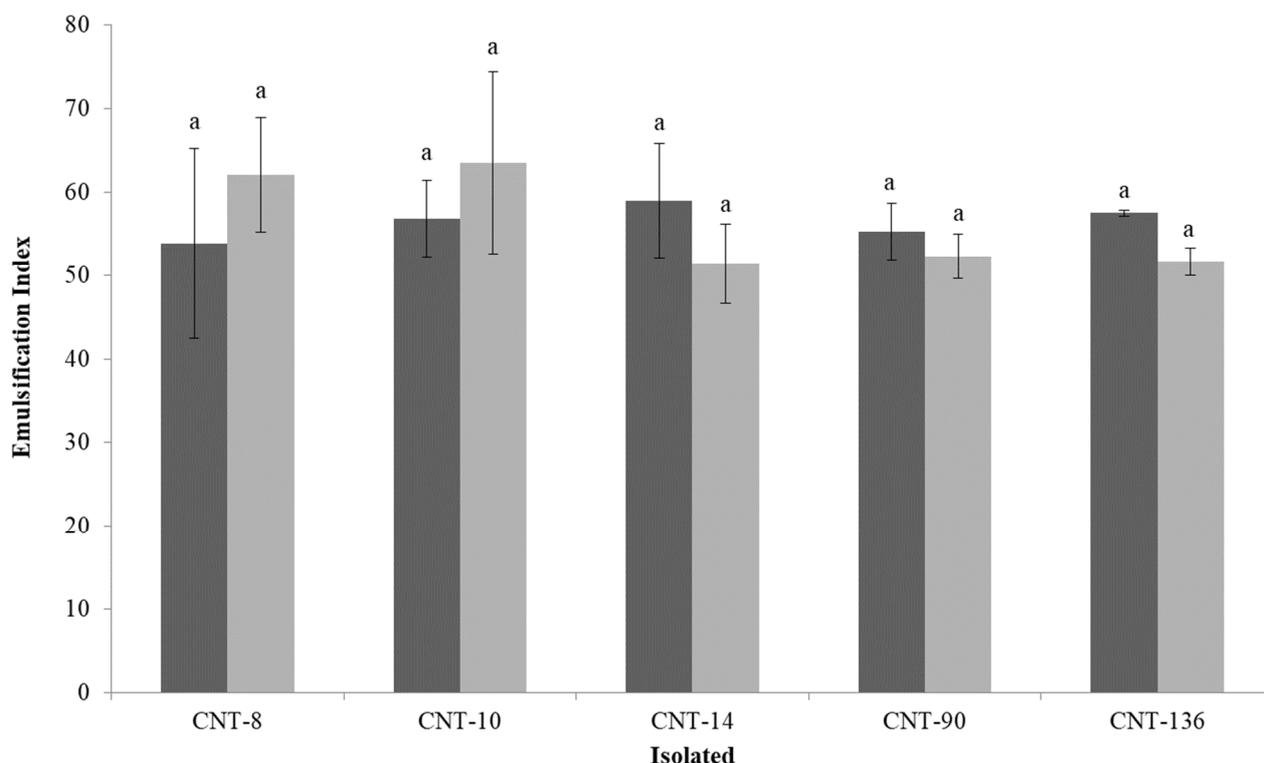


Figure 3. Emulsification index of bioemulsifiers produced by *Yarrowia lipolytica* from growth using residual glycerol as a carbon source. (■ - 24h and ■ - 48h).

The *P. aeruginosa* biosurfactant production using glycerol (6.0%) obtained from a biodiesel production process as a carbon source was studied by (Sousa et al., 2011). The result obtained by authors evidenced a 64.0% emulsification index and a 45.7% reduction in surface tension. Fontes et al. (2012) noted the high emulsification index produced in the medium containing crude glycerol, can be justified obtained from the biodiesel production process, which in turn may have oil and fatty acid impurities, which are molecules that can be absorbed easily by *Y. lipolytica* being incorporated into the nonpolar part of the biosurfactant molecules giving it emulsifying properties.

Conclusion

Bioemulsifier production using cheap and abundant raw material is a plus, due to cost savings, this can make the production process cheaper and make bioproducts competitive with synthetics. Highlighting the importance of results that can be considered satisfactory using waste, making it attractive because it is used in its raw form. These results have relevance for various applications of bioemulsifiers that is important in solubilizing hydrophobic compounds in the bioremediation process.

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