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Screening, Production and Characterization of Biosurfactants from Caatinga´S Filamentous Fungi

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Abstract: *The Caatinga biome may be a source for obtaining metabolites with unique features, such as biosurfactants of microbial origin, amphipathic molecules capable of reducing the surface tension and emulsify hydrocarbons. This study aimed to production and characterization of biosurfactants produced by Caatinga's filamentous fungi, collected in Sumé city, stored in a collection at CDSA/UFCG. In this research, 10 filamentous fungi were evaluated in relation to emulsifier capacity, totaling six specimens (60%) presented results for Emulsification Index (EI), stability after 24 hours and Emulsification Activity (EA), with emphasis for metabolic liquids produced by fungi CDSA17, CDSA54 and CDSA71, from the genus Aspergillus, that were also evaluated under extreme conditions of temperature and pH, with higher EI at temperature of 60 °C, pH 7.0 and salt concentration 10%. In factorial design 2², in duplicate, analyzing variable of shaking (100 and 200 rpm) and glucose concentration (15 g/L and 25 g/L), tested filamentous fungi showed different behaviors and higher IE were obtained under conditions of 100 rpm and glucose concentration of 15 g/L. According to the above, it is clear that Caatinga's filamentous fungi have potential to produce biosurfactants.*
Keywords: *Aspergillus, Bioprospection, Emulsification, Factorial Design, Surface-Active Substances.*

I. INTRODUCTION

The Caatinga is the source of biological diversity biotechnological processes with the potential to open the extreme conditions that the microorganisms are adapted to range of pH, salinity, temperature and nutrients [1].

Among the compounds of microbial biological surfactants or biosurfactants has aroused interest on numerous applications in industrial, economic and environmental level. Forming a group of agents that act at the interface of emulsions and the ability to lower the surface and interfacial tension of liquids [2], the biosurfactants are amphipathic molecules employed to increase the capacity of emulsification, the interaction of oil/water and accelerate the degradation of hydrocarbons by microorganisms [3]. Biological surfactants have the advantages such as low toxicity, biodegradability, efficiency at extremes pH, temperature and salinity [4].The biosurfactant production conditions are determined according to the microorganism and the substrates utilized [5]. Carbon and nitrogen sources, well as factors such as pH, temperature, agitation, and the conduction of the process are extremely important to the quantity and quality of the compost produced [6]. The ability to form the emulsion, reduction of surface and interfacial tension and determination of the Critical Micelle Concentration is used as a means of determining the synthesis and efficiency of the biosurfactant produced by microorganisms [4].

In view of this biological diversity in the Caatinga, the possibility of alteration in the metabolism of the microorganisms outstanding to environmental conditions which are exposed and advantages of biosurfactants regarding synthetic surfactants, this study was developed to the biotechnological exploitation of the microorganisms of the Caatinga for production and characterization of these compounds through filamentous fungi.

II. MATERIALS AND METHODS

2.1 Microorganisms

The microorganisms used are part of the collection of filamentous Fungi in the Caatinga of the Centre for Sustainable Development in the Semiarid of the Federal University of Campina Grande (CDSA/UFCG), isolated from successive collections of fungi in the soil and leaves of plants Caatinga. We used 10 randomly

selected specimens, named as CDSA06, CDSA17, CDSA20, CDSA43, CDSA50, CDSA54, CDSA71, CDSA74, CDSA103 and CDSA107. The fungi that produce biosurfactants were identified through the study of different fungal structures, following the methods described by the National Health Surveillance Agency [7].

2.2 Production of biosurfactants

Pre-inoculum was carried out in 50 mL of YEPD liquid culture medium from the fungal spores obtained from samplings, the microorganisms were incubated overnight under orbital agitation of 150 rpm, 37ºC, at that moment 1 mL was transferred to a new cultivation in flasks, containing 50 mL medium YEPD, under the conditions of 150 rpm agitation, for 96 hours, at 37°C. After the incubation period, the vials were subjected to vacuum filtration, in a 25µm membrane, to obtain of the liquid metabolic free of cells.

2.3 Determination of the presence of biosurfactants

2.3.1 Emulsification Index (EI)

The emulsification Index (EI) was determined using 2 mL of metabolic liquid and 1 mL of kerosene, and then homogenized by stirring, for 2 minutes, at room temperature (25°C) [8]. The tests were performed in duplicate and the index was calculated according to Equation 1. The formed liquid emulsion was maintained at room temperature, and after 24 hours it was checked for stability [9]. In which *He*: height of the emulsion; *Ht*: total net height.

$$EI(\%) = \frac{H_e}{H_t} x\ 100 \quad (1)$$

2.3.2 EmulsificationActivity (EA)

The Emulsification Activity was determined using 2 mL of metabolic liquid, 2 mL of 0.1M sodium acetate buffer (pH 3.0) and 1 mL of kerosene. The mixture was stirred, for 2 minutes, followed by standing for 10 minutes, afterward the emulsion was removed through Pasteur pipette and the analysis carried out in a spectrophotometer, at 540nm [10]. The result was expressed in Emulsification Activity Unit (EAU) according to Equation 2. In which *EAU*: Emulsification Activity Unit; *Abs*: Absorbance.

$$EAU = Absx\ 2 \quad (2)$$

2.4 Determination of effect of temperature, pH and salinity on the activity of the biosurfactant

The liquid metabolic, free of cells, were maintained at temperatures of 3°C and 60°C, for 10 minutes, to set the stability of the biosurfactant. After temperature treatment, each solution was standardized to room temperature to measure of EI. The temperature that metabolic liquids showed higher indices was studied through checking after 20 minutes interval and the emulsion stability after the period of 60 minutes.

To determine the effect of pH over the emulsification index, the pH of the liquid containing the metabolic biosurfactant was adjusted to 5.0; 7.0 and 9.0, using sodium hydroxide base (NaOH) and hydrochloric acid (HCl). After 30 minutes, EI was determined. The biosurfactants present in liquids metabolic, free of cells, were evaluated for emulsion stability at the concentration of 5% and 10% NaCl. After 30 minutes of incubation, EI was determined.

2.5 Factorial designs

The biosurfactant production was investigated by varying agitation and glucose concentration for its effects and interactions in the synthesis of this compound. A 2^2 factorial design, in duplicate, with center point was performed to analyze the influence of these parameters on the response Emulsification Index (%).All designs were developed and analyzed with the help of software Statistica 7.

III. RESULTS

3.1 Screening and production of biosurfactants

From 10 specimens from the collection of fungi Caatinga, according to Table 1, six of them (60%) were considered potential producers of biosurfactants, after analyzed by the ability to produce, and emulsion stability after 24 hours, and emulsifying activity. Only metabolic liquids produced by CDSA06 and CDSA107 fungi showed no emulsion formation, therefore it was not possible to carry out stability tests and EA. The fungi CDSA20 and CDSA43 were able to form emulsion, in duplicate, but not remained stable over 50% after 24 hours. The others fungi produced biosurfactants with stability ranging from 56-83%, highlighting the CDSA17 which exhibited stability of 83%. The biosurfactants produced by CDSA17 specimens CDSA54 and CDSA71 worth mentioning to present parameters Emulsification Index, stability after 24 hours and Emulsification Activities superior to the others tested.

The cultures of the biosurfactants producers, fungi CDSA17, CDSA54 and CDSA71 were observed under an optical microscope in 40x magnification. The Filamentous fungi CDSA17, CDSA54 and CDSA71 showed similar macroscopic and microscopic characteristics of the genus *Aspergillus*, with filamentous mycelium and colonies initially white, with color varying over growth, according to the species.

Table 1. Results of EI and EA performed to verify the production of biosurfactants by filamentous fungi Caatinga

Fungi	Emulsification Index (%)	Stability (%)	Emulsification Activity (540 nm)
CDSA06	0	0	0
CDSA17	26.47 ± 0.1	22.04 ± 2.1	0.98 ± 0.0
CDSA20	29.43 ± 1.2	13.19 ± 1.5	*
CDSA43	13.26 ± 2.1	05.88 ± 0.0	*
CDSA50	22.22 ± 2.0	14.71 ± 0.6	0.04 ± 0.0
CDSA54	25.82 ± 3.3	14.96 ± 1.7	1.19 ± 0.2
CDSA71	25.00 ± 2.1	16.17 ± 2.1	0.99 ± 0.3
CDSA74	29.21 ± 1.5	23.01 ± 6.0	0.12 ± 0.1
CDSA103	17.64 ± 8.3	14.70 ± 4.2	0.30 ± 0.0
CDSA107	0	0	0

* Metabolic liquids were not tested because not having a stability > 50 %.

3.2 Effect of temperature, pH and salinity on the biosurfactant's activity

Tests conducted with the biosurfactant produced by specimens CDSA17, CDSA54 and CDSA71 indicated that, when subjected to temperature extremes (low/ high), the Emulsification Index showed better results when compared to the tests performed at room temperature (25°C) (Table 1). The biosurfactants from CDSA17 and CDSA71 showed EI higher when exposed to 60°C (Table 2). Because of this an assessment, of stability was checked at 60°C at 60 minutes (Figure 1).

Table 2. Stability of biosurfactants at temperatures of 3°C and 60°C

Fungi	Emulsification Index (%)	
	3ºC	60 ºC
CDSA17	25.71 ± 0.1	29.41 ± 0.5
CDSA54	28.57 ± 0.5	22.86 ± 0.6
CDSA71	26.47 ± 0.1	30.00 ± 1.0

Most of the specimens showed small variations of the Emulsification Index over time, however without reducing the EI from the values obtained in the pattern (Fig. 1). The biosurfactants of specimens CDSA17 and CDSA71 gradually increased the Emulsification Index after 20 minutes of exposure to the heat treatment, but after 40 minutes the EI from CDSA71 was reduced. The CDSA54 emulsion was stable after 40 minutes. Evaluating the effect of pH on the biosurfactant produced, it is observed that Emulsification Index obtained in the range pH 5.0 and 7.0 were near the pattern for CDSA54 and CDSA71, the alkaline pH of the emulsion CDSA54 was severely reduced. For the three tested specimens, biosurfactants were stable at pH 7.0 with EI is about 20 to 32.5%, as showed in Fig. 2.

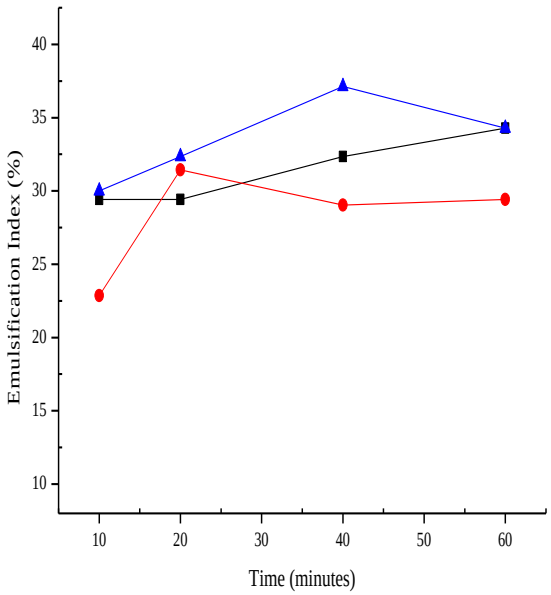


Figure 1. Emulsion stability of fungal specimens (■) CDSA17, (●) CDSA54 and (▲) CDSA71, up to 60°C

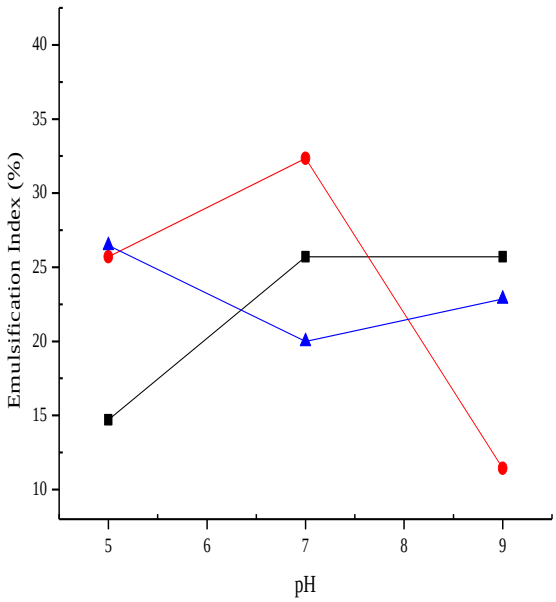


Figure 2. pH effect on the IE of biosurfactants from specimens (■) CDSA17, (●) CDSA54 and (▲) CDSA71

The metabolic liquid specimens were tested to EI behavior in concentrations of 5% and 10 % salinity (Table 3). The results presented in Table 3 suggest that biosurfactants present in the metabolic liquids from

CDSA17 and CDSA71 were not stable at a concentration of 5% NaCl, but formed emulsion at concentration of 10%. As the metabolic liquid produced by CDSA54 presented Emulsification Index in two concentrations, greater EI (23.53%) at salinity of 5%, demonstrating that the emulsion CDSA54 is more stable at 5% concentration, but still emulsifying capacity (60.7%) at 10% NaCl.

Table 3. Determination of the effect of NaCl concentration on EI

Fungi	Emulsification Index (%)	
	NaCl 5%	NaCl 10%
CDSA17	0	15.15 ± 0.4
CDSA54	23.53 ± 1.2	14.29 ± 0.2
CDSA71	0	20.0 ± 0.7

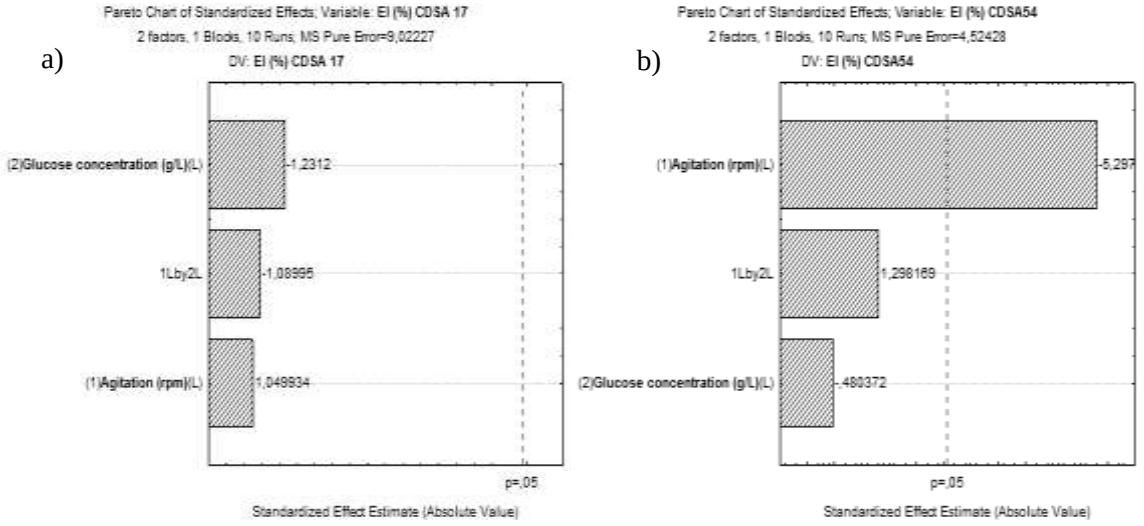
3.3 Factorial design to optimize biosurfactant production

Table 5 displays the tested specimens and the results of the experimental model performed to optimize the production of biosurfactant. All organisms displayed IE exposed in culture conditions, but CDSA17 fungus obtained the slight responses, with EI lower than standard (Table 1). The specimens CDSA54 and CDSA71 exhibited the highest Emulsification Index in conditions of 100 rpm and glucose concentration of 15 g/L (test 1 and 5), with EI higher than standard.

Fig. 3 illustrates the Pareto´s Diagrams, with 95% confidence limit for the estimated effects on EI dependent variable. It is possible to observe that in factorial design developed for the specimen CDSA17 (Fig. 3–a) independent variables agitation and glucose concentration and the interactions between them did not exert statistically significant effects on the Emulsification Index. In the analysis of the results for the CDSA54 fungus (Fig. 3-b) the variable agitation significantly influenced the EI, presenting effect with a negative sign, indicating for this specimen, agitation of 100 rpm is better for the production of biosurfactants. In the analysis of Pareto diagram (Fig. 3-c), for CDSA71 fungus, is possible to observe that the interaction between the variables agitation and glucose concentration was statistically significant in Emulsification Index. The variable glucose concentration also showed a statistically significant effect on EI.

Table 5. Results of the factorial design 2^2 to determine the best growing conditions in the biosurfactants production by adopting in response the Emulsification Index

Test	Independent variables		Dependent variable Emulsification Index (%)		
	Agitation (rpm)	Glucose concentration (g/L)	CDSA17	CDSA54	CDSA71
1	100 (-1)	15 (-1)	09.09	26.47	29.41
2	200 (+)	15 (-1)	17.65	18.18	23.53
3	100 (-1)	25 (+1)	14.71	25.71	18.18
4	200 (+)	25 (+1)	11.77	21.21	22.12
5	100 (-1)	15 (-1)	17.65	30.30	26.47
6	200 (+)	15 (-1)	18.18	18.75	20.59
7	100 (-1)	25 (+1)	11.43	25.71	21.21
8	200 (+)	25 (+1)	14.20	18.18	23.55
9	150 (0)	20 (0)	26.40	23.53	26.47
10	150 (0)	20 (0)	26.47	28.12	23.53



c)

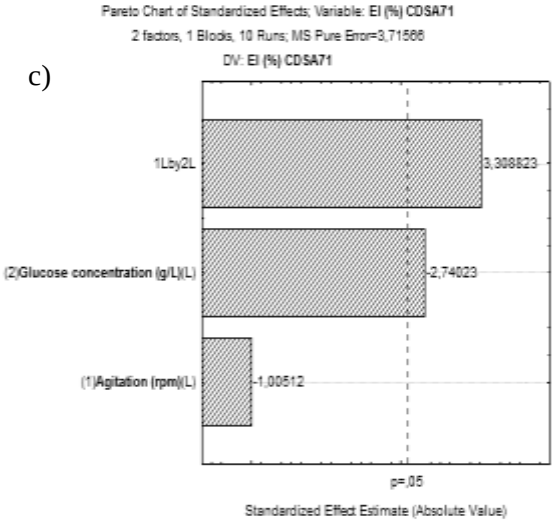


Figure 3. Pareto´s Diagram of the standardized effects for Emulsification Index (EI %) for the full factorial design 2^2 used to optimize biosurfactants production by filamentous fungi a) CDSA17, b) CDSA54 and c) CDSA71

IV. DISCUSSION

For the reason that the ability of biosurfactants produced by filamentous fungi Caatinga to form emulsion, these substances tend to present importance in the composition of products from various industry sectors, such as cosmetics, food and even detergents or cleaning agents [11; 12] and the industry can use these compounds as emulsifying agents, foaming, wetting and solubilizing agents [13]. The results obtained in characterization of the biosurfactants demonstrate the ability of biosurfactants synthesized by fungi Caatinga to maintain stable emulsions at high temperature for a certain period of time, with possible application in industries where heating to achieve sterilization is main importance [14]. Similar results to this study were observed in biosurfactant stability study produced by *Aspergillusfumigatus* grown in solid state fermentation, analyzing Emulsification Activity biosurfactant when subjected to pH 5.0 provided increased EA, compared to assays using pH 9.0, with biosurfactant also presenting greater stability at pH 7.0 compare to the pattern [15]. Biosurfactant produced by *A. flavus* was stable in the pH range of 3-12 and temperature of 80 °C [16].

Several studies have found that increased production of biosurfactants is achieved when there is an increase in the agitation speed to affect the mass transfer of oxygen molecules and components of the medium [17; 18]. However, for some microorganisms such increase may cause a shearing effect on cell wall, causing mechanical stress in the cells and reducing the synthesis of biosurfactant [19]. The *Bacillus* species showed better biosurfactant production when cultured in low concentrations of sucrose [20]. 3% glycerol as a carbon source concentrations reduced the biosurfactant synthesis by bacteria *Pseudomanasstutzeri* BK- AB12 [21].

V. CONCLUSION

The biotechnological exploration of Caatinga´s microorganisms shows that is possible to detect filamentous fungi with the potential for producing biosurfactants. The specimens CDSA17, CDSA 54 and CDSA71 belong to the genus Aspergillus produced compounds able to emulsify hydrocarbon and maintain emulsion stability at a temperature of 3 °C and 60 °C, pH 7.0 and salt concentration of 10%, allowing application in industrial processes in extreme conditions. The use of experimental design has proved to be an applicable tool to optimize biosurfactants production by CDSA54 and CDSA71 fungi. Therefore, Caatinga´s fungi are a new source for obtaining biosurfactants with emulsifying capacity indicating future prospects for application in industrial sectors such as pharmaceutical, food and refineries, and bioremediation of contaminated areas.

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