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Development and improved selected markers to biosurfactant and bioemulsifier production by *Rhizopus* strains isolated from Caatinga soil

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This study screened four *Rhizopus* species as biosurfactant producers using different markers. First of all, *Rhizopus* spp. UCP 1607 was identified as *Rhizopus arrhizus* by morphological and molecular methods. The production of biosurfactant/bioemulsifier was investigated by submerged fermentation using soybean post-frying oil (5% v/v) and sodium glutamate (1% w/v) medium. The primary markers' hemolysis and parafilm M tests showed that *R. arrhizus* UCP1607 strain exhibited higher hemolytic activity (49 mm of clear zone) on sheep blood agar and a larger drop diameter (12 mm) on parafilm hydrophobic surface. The experimental results showed the most promising biosurfactant production by *R. arrhizus* UCP 1607 strain led to a reduction of surface tension (31.8 mN/m) and the diameter of the oil-spreading covered an area of 66.4 cm². The strains *Rhizopus microsporus* var. *chinensis* UCP1296, *R. microsporus* var. *microsporus* UCP1304, and *R. arrhizus* UCP1607 were capable of forming stable emulsions corresponding to 91.7, 94.8, and 82.6%, respectively in crude oil.

Key words: Tensio-active agent, bioemulsifier, screening of *Rhizopus* strains, submerged fermentation.

INTRODUCTION

Caatinga is a biome that comprises an extensive semi-arid area of 969.589,4 km², located in the Northeast of Brazil. A prominent feature of the semi-arid region of Caatinga is the climate markedly characterized by severe environmental conditions where high temperatures with the minimum above 15°C and the maximum around...
40°C, intense insolation, scanty water resources, and the annual rainfall in the area is estimated to be lower than 1000 mm, leading to prolonged periods of serious drought. Moreover, the predominantly shallow soils present low natural fertility (Menezes et al., 2012; Silva et al., 2015). These environmental conditions of the semi-arid region of Caatinga biome have a direct influence on soil microbial life. Thus, microorganisms that survive in these stressful conditions do develop adaptive mechanisms of response, synthesizing appropriate metabolites (Gorlach-Lira and Coutinho, 2007; Silva et al., 2015).

Studies aiming to explore the biotechnological potential of genus Rhizopus have demonstrated that species of this genus are able to produce different types of compounds of an enormous industrial importance, namely enzymes (Freitas et al., 2014), organic acids (Abe et al., 2007), chitin, and chitosan (Berger et al., 2014) including biosurfactant (Freitas Silva et al., 2012).

Biosurfactants are products of the metabolism of living cells especially of bacteria, yeasts, and filamentous fungi that may be produced extracellularly or as part of cell membranes (Mulligan, 2005).

Structurally, biosurfactants are amphiphatic molecules possessing hydrophobic and hydrophilic domains as basic information according to Desai and Banat (1997). Their complex structural organization gives them important physico-chemical properties such as lowering surface and interfacial tensions between immiscible phase systems, promoting the formation of micelles through which hydrophobic compounds can be solubilized in water or vice-versa (Fracchia et al., 2015). In addition, these compounds are known to be efficient dispersing and emulsifying agents, exhibit high wetting and foaming abilities, and display low critical micelle concentration (Mnif and Ghribi, 2015). These properties make biosurfactants molecules with a wide range of practical applications in the bioremediation of contaminated environments, enhanced oil recovery, as ingredients in the food processing industry, cosmetics and pharmaceutical industry (Makkar et al., 2011; Mnif and Ghribi, 2015).

The natural origin of these molecules turn them more interesting compounds, along with non-low toxicity, high biodegradability, effectiveness at extreme conditions (pH, temperature, and salinity), biocompatibility, and specificity in their function (Makkar et al., 2011).

Due to their advantages and numerous possible uses in different areas, microbial surfactants have been the central point of diverse studies aiming to identify potential microorganism producers of these molecules (Walter et al., 2010). However, the majority of screening studies have been carried out using bacteria (Kebbouche-Gana et al., 2009; Nwaguma et al., 2016; Joy et al., 2017; Batool et al., 2017), and outgrows by far those evaluating the fungi producing potential (Sari et al., 2014; Lodha et al., 2016). Till date however, the need for discovery of biosurfactant producing microorganisms capable of inhabiting environments featured by adverse typical conditions such as extreme salinity, higher temperatures, and scanty humidity is still enormous (Techaoei et al., 2007; Kebbouche-Gana et al., 2009; Kiran et al., 2010). Hence, the objective of the current study was to evaluate the potential of Rhizopus strains in the production of biosurfactants using different screening methods (Kiran et al., 2010; Sari et al., 2014).

**MATERIALS AND METHODS**

**Micro-organisms**

*Rhizopus* strains from the Caatinga soil used were: *Rhizopus arrhizus* var. *arrhizus* UCP 1295 and *Rhizopus microsporus* var. *chinensis* UCP 1296 and var. *microsporus* UCP 1304 and were kindly provided by the culture collection, UCP (Catholic University of Pernambuco), Recife-PE, Brazil which is registered in the World Federation for Culture Collections (WFCC).

**Fungus isolation**

The new *Rhizopus* species strains were isolated from Caatinga soil sample collected in Natal, Rio Grande do Norte state, Northeast of Brazil and were used in the following media (g/L): wheat germ agar medium (wheat germ 15, glucose 5, and agar 15, supplemented with chloramphenicol 0.1), malt extract agar (MEA; malt extract 20 and agar 20). S. dextrose agar (SDA; peptone 10, glucose 40, and agar 20). The isolation of the fungus was carried out by soil sprinkling technique according to Benny (2008). Briefly, 5 mg of soil sample was weighed using a precision balance and then were spread onto wheat germ agar medium plates and incubated at 28°C until sporulation. Afterwards, using a sterile syringe mature sporangiospores, they were transferred directly from the colonies to MEA plates and then incubated at 28°C for 7 days. Pure culture of the isolate was maintained on SDA slants and stored at 4°C in a refrigerator. Transfers were done to fresh SDA slants, each three months to maintain the isolate viable.

**Morphological and molecular identification**

The macroscopic and microscopic identification was conducted according to Zheng et al. (2007). The macroscopic morphology (colony size, aspect and color) was attained by naked eye examination of 5 to 7 days old culture grown on potato dextrose agar (PDA) medium (g/L: peeled potato 200, dextrose 20, and agar 15). The microscopic morphological observed by light microscopy using Lactophenol Cotton Blue staining and distilled water.

The genomic DNA was extracted from mycelium grown on PDA for 72 h, at 28°C by the cetyltrimethylammonium bromide (CTAB) DNA Extraction Protocol method, adapted from Doyle and Doyle (1991). The ribosomal DNA ITS1-5.8S-ITS2 and LSU regions were amplified by polymerase chain reaction (PCR) on a Peltier PTC-100® thermocycler (MJ Research, Inc., USA) in a total volume of 25 μL of sample. The rDNA ITS regions were amplified using primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS2 (3'-GCTGCGGTTGCTTATACGACG-5') (White et al., 1990). The D1/D2 LSU region of the rDNA was sequenced using primers NL1 (5'-GCATATCATAATAGGGAGGA-3') and NL4 (5'-GGTTCCGGTGTATTCAAGCAGGTGC-3') (O’Donnell, 1992). The amplicons were purified with PureLink-PCR Purification Kit C/500xn.
Screening for biosurfactants production

Primary screening: Hemolysis and parafilm M tests

Preliminary identification of the potentially biosurfactant-producing *Rhizopus* strains was performed by the hemolytic activity test (Satpute et al., 2010). Spores of *Rhizopus* strains were inoculated on the central part of the agar plate containing 5% (v/v) of defibrinated sheep blood and incubated at 28°C for four days. The experiments were monitored for observation of hemolytic activity which was detected by appearance of clear zone on blood agar plate.

Parafilm M assay is a rapid and simple test that does not require specialized equipment and can be carried out with small sample volumes. The test consisted of placing 25 μL of mycelia-free metabolic liquid on hydrophobic surface of the parafilm M strip. The shape of the drops was examined after 1 min and its diameters were measured using a caliper. The presence of the surface active compounds in the mycelia-free metabolic liquid was detected by the flat shape of the drop, while the semispherical shape indicates the absence of biosurfactant/bioemulsifier. The medium without microorganism was used as control (Sari et al., 2014).

Biosurfactant/Bioemulsifier production

The strains *R. arrhizus* var. *arrhizus* UCP 1295, *R. microsporus* var. *chinesis* UCP 1296, *R. microsporus* var. *microsporus* UCP 1304, and *Rhizopus* spp. UCP 1607 were grown on PDA for 96 h at 28°C. Spore suspensions were prepared in the sterile water and adjusted to 10⁷ spores/mL, and 5% of suspensions were inoculated in Erlenmeyer flasks containing 100 mL of the medium constituted by soybean post-frying oil (5% v/v), sodium glutamate (1% w/v), and salt solution (g/L): (NH₄)₂NO₃ 1.0, KH₂PO₄ 0.2, and MgSO₄.7H₂O 0.2, and the pH was adjusted to 5.5. The flasks were incubated in orbital shaker at 150 rpm, at 28°C during 96 h. The net metabolic liquid containing biosurfactant was obtained by filtration followed by centrifugation (10,000 × g for 15 min), and was used for secondary screening.

Secondary screening

Surface tension determination

The measurement of surface tension in the mycelia-free broth was performed using an automatic Tensiometer (model Sigma 70 KSV Ltd, Finland) utilizing the Du Nouy ring method as described by Kuyukina et al. (2001). The results were reported as the average of measurements in triplicate.

Oil spreading assay

In order to determine the biosurfactant dispersing ability, oil spreading test was applied (Andrade Silva et al., 2014). Distilled water (40 mL) was inserted in a Petri dish (15 cm of diameter), and this was followed by addition of 1.0 mL of burnt motor oil onto water layer surface. After that, 0.5 mL of metabolic liquid (A), 0.5 mL of commercial detergent (B), 0.5 mL of chemical surfactant SDS (C), and 0.5 mL of distilled water (D) were placed in the center of the oil film. The presence of the biosurfactant/bioemulsifier in the mycelia-free broths was detected by the spreading of oil resulting in the formation of oil displacement areas. The clear zone diameters were measured and the respective oil displacement areas (ODA) were determined and expressed in cm² using the equation that follows. The experiments were performed in triplicate.

\[ ODA = 3.14 \times r^2 \]

Emulsification index (E₂₄)

The emulsifier properties of the biosurfactant in crude extracts produced by *Rhizopus* strains were evaluated by emulsification index assay. For determination of emulsification index, 1.0 mL of mycelia-free metabolic liquid containing biosurfactant and 1.0 mL of burnt motor oil were mixed together in a test tube, and then homogenized vigorously for 2 min at room temperature (25°C). After 24 h, measurements were performed through the equation:

\[ E_{24} (\%) = \frac{He}{Ht} \times 100 \]

where \( He \) = emulsion height; \( Ht \) = mixture total height (Liu et al., 2013).

RESULTS AND DISCUSSION

Isolation, phenotypic and molecular identification

The isolation of *Rhizopus* spp. UCP 1607 from the Caatinga semi-arid region soil sample was accomplished on basis of colony morphology (Figure 1). The isolate
showed rapid growth on PDA plates at a temperature of 28°C, covering the entire Petri plate of 9 cm in diameters with abundant mycelia, and the colonies were cottony, initially white and later turned gray blackish (A). Under light microscope straight and opposite sporangiophores arising from rhizoids were observed (B). Rhizoids finger-like branched (C). Straight to substraight, 2 to 3 in groups and opposite sporangiophores arising from rhizoids, 285 to 840 to 1000 µm long, 8 to 17 µm diameters. Sporangia black, globose to slightly depressed globose, 72 to 168 µm diameter. Columellae subglobose, 50 to 114 × 78 to 149 µm, light grayish-brown. Sporangiospores ovoid, the mostly regular in shape and size, 5 to 7 × 4 to 6.5 µm, light gray the solitary (D). The fungus was identified as belonging to *R. arrhizus* (Zheng et al., 2007).

Species of *Rhizopus* are worldwide distributed, inhabiting different environments (Ribes et al., 2000), so they may be isolated from soil, dung, decaying organic material and mature fruits (Santiago et al., 2013), and a variety of food products (Abdel-Hafez, 1984). Some species of this genus live as pathogens causing diseases in humans, animals and plants (Santiago et al., 2013).

There are various studies on isolation and assessment of diversity of tropical areas filamentous fungi including Brazil (Siqueira and Brussaard, 2006; Cavalcanti et al., 2006). However, only few reports referred to isolates from semi-arid environments (El-Said and Saleem, 2008; Grishkan and Nevo, 2010). In this context, little is known about filamentous soil mycota of the Caatinga biome.

Considering this fact, Cavalcanti et al. (2006) studied the diversity of soil filamentous fungi in Xingó, state of Bahia, a region with typical Caatinga ecosystem. Among Zygomycota, two *Rhizopus* spp. were isolated and identified as *R. microsporus* var. *chinensis* and *R. microsporus* var. *microsporus*. Santiago et al. (2013) worked from soil samples of three different semi-arid areas of the state of Pernambuco to evaluate the distribution of Mucorales order. These authors reported the *R. microsporus* var. *microsporus* (10.19%) and *R. arrhizus* var. *arrhizus* (7.41%) as one of the most frequent genus in the three areas. In this study, *Rhizopus stolonifer* and *R. microsporus* var. *chinensis* were isolated as well. Oliveira et al. (2013) assessed the diversity of filamentous fungi from soil in the same state and identified same species of *Rhizopus* (*R. microsporus* var. *microsporus* and *R. arrhizus*).

It was observed that molecular identification of the isolate, using the nucleotide sequence found was compared to those deposited in National Center for Biotechnology Information (NCBI) website using the BLAST program. The results identified homology of 95% of similarity to *R. arrhizus*. In the current study, the phenotypic characteristics of the *R. arrhizus* matched with the molecular analysis for the definitive identification of the fungus. Different rDNA regions have been frequently used for the identification of Mucorales. Moreover, the 18S regions of the small ribosomal subunit, the region of the internal transcribed spacer (ITS), and the large ribosomal subunit (LSU) region are the most commonly used as markers (Kasai et al., 2008; Bellemain et al., 2010; Yang et al., 2016; Ziaee et al., 2016).

### Detection of biosurfactant-producing *Rhizopus* strains

According to Thavasi et al. (2011), microorganisms with positive hemolytic activity for production of biosurfactants show a clear zone in the blood agar plates. In this context, only the strain *Rhizopus* spp. UCP 1607 produced extracellular compound during the radial growth on the blood agar and formed a higher clear zone diameter (40 mm), after 72 h of incubation. The hemolytic activity demonstrated by the strain was superior to that presented by *Aspergillus* species MSF1 that caused the appearance of a clear zone with a diameter of 7 mm in blood agar medium as described by Kiran et al. (2010). The hemolytic activity was employed by several authors as initial criterion for selection of biosurfactant producers (Batool et al., 2017; Nwanguma et al., 2016). However, Satpute et al. (2010), recommend the use of more than one screening method for detection of biosurfactant-producing microorganisms.

Parafilm test and surface tension determination are both physical methods widely applied for identification of biosurfactant-producing microorganisms (Sari et al., 2014; Korayem et al., 2015). The results from parafilm M assay showed that *Rhizopus* strains tested in this work were able to produce biosurfactants with different surface-active properties, since the droplet diameter of the metabolic liquid of each strain was larger, compared with fresh culture medium (Table 1). However, the best

<table>
<thead>
<tr>
<th>Strain</th>
<th>Parafilm M test (mm)</th>
<th>Surface tension (mN/m)</th>
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<tbody>
<tr>
<td><em>Rhizopus arrhizus</em> var. <em>arrhizus</em> UCP 1295</td>
<td>8</td>
<td>35.0</td>
</tr>
<tr>
<td><em>Rhizopus microsporus</em> var. <em>chinensis</em> UCP 1296</td>
<td>10</td>
<td>33.3</td>
</tr>
<tr>
<td><em>Rhizopus microsporus</em> var. <em>microsporus</em> UCP 1304</td>
<td>9</td>
<td>34.7</td>
</tr>
<tr>
<td><em>Rhizopus</em> spp. UCP 1607</td>
<td>12</td>
<td>31.8</td>
</tr>
<tr>
<td>Fresh medium (control)</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>
Figure 2. Tensioactivity of crude biosurfactants of the *Rhizopus* strains on parafilm M hydrophobic surface.

Table 2. Oil displacement area by biosurfactants/bioemulsifiers produced by different *Rhizopus* strains compared with chemical surfactant and commercial detergent.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Oil displacement area, ODA (cm²)</th>
</tr>
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<tbody>
<tr>
<td><em>Rhizopus arrhizus</em> var. <em>arrhizus</em> UCP 1295</td>
<td>22.0</td>
</tr>
<tr>
<td><em>R. microsporus</em> var. <em>chinensis</em> UCP 1296</td>
<td>56.7</td>
</tr>
<tr>
<td><em>R. microsporus</em> var. <em>microsporus</em> UCP 1304</td>
<td>39.6</td>
</tr>
<tr>
<td><em>Rhizopus</em> sp. UCP 1607</td>
<td>66.4</td>
</tr>
<tr>
<td>Commercial detergent</td>
<td>44.2</td>
</tr>
<tr>
<td>Chemical surfactant (SDS)</td>
<td>75.4</td>
</tr>
<tr>
<td>Distilled water</td>
<td>Negative</td>
</tr>
</tbody>
</table>

results were exhibited by the new strain of *R. arrhizus* UCP 1607 forming largest drop (12 mm diameter) on the hydrophobic surface (parafilm M strip) (Figure 2).

The surface tension of the cell free broth from *R. arrhizus* UCP1607 reached the lowest value (31.8 mN/m). Correlations between the drop diameters and the reduction of surface tension and the drop spreading value from *Rhizopus* strains were observed (Table 1).

Sari et al. (2014) evaluated the capability of isolates of *Pseudozyma* strains for biosurfactants production, and found the surface tensions varying from 35.8 to 44.3 mN/m. Therefore, it was concluded that the biosurfactants produced by all *Rhizopus* strains were their primary metabolite, due to the production of growth-associated biosurfactant.

According to Sharma et al. (2016), a microorganism is considered a good surface-active compounds producer if its net metabolic liquid is able to reduce the surface tension of water from 72 to 35 mN/m or below this value. Similar criterion for biosurfactants-producing microorganism detection was applied by Ariech and Guchi (2015), considering surface-active potential biomolecule reduced the surface tension below 40 mN/m.

**Dispersing capacity and emulsification activity of the *Rhizopus* strains**

The oil displacement assay requires no specialized equipment, and also the method is rapid and simple which can be undertaken with small volumes of sample (Walter et al., 2010). Table 2 shows the results for dispersing ability of the crude biosurfactant extracts produced by *Rhizopus* strains. Thus, significant oil displacement activities were demonstrated by biosurfactants produced by *R. microsporus* var. *microsporus* UCP1304 and *R. microsporus* var. *chinensis* UCP1296 corresponding to 39.6 and 56.7 cm² of oil displacement areas, respectively. However, the biosurfactant produced by *R. arrhizus* UCP1607 exhibited excellent potential in the dispersion of burnt motor oil on water surface that resulted in 66.4 cm² of oil displacement area (ODA). The results showed that the oil displacement area of the biosurfactant produced by *R. arrhizus* UCP1607 (Figure 3A) was superior to dispersion induced by commercial detergent (44.2 cm²) (Figure 3B). Synthetic surfactant dodecyl sulphate (SDS) showed the best dispersion (75.4 cm²) (Figure 3C) as positive control, as well as the negative control in which the burnt motor oil with distilled water was used (Figure 3D).

The data obtained in this study using *Rhizopus* strains were superior to dispersion capacity of the biosurfactant produced by *Pleurotus ostreatus* (12.56 cm²) of oil displacement area (Velioğlu and Öztürk Ürek, 2015), and the bioemulsifier production by *Cunninghamella echinulata* (37.36 cm²) (Andrade Silva et al., 2014).

According to Walter et al. (2010), the emulsification index is a reliable method for detection of bioemulsifier producers. *Rhizopus* strains showed emulsifying capacity of biosurfactants produced (Figure 4).
Figure 3. Oil displacement area (ODA) for: (A) Biosurfactant produced by *Rhizopus arrhizus* UCP1607, (B) Commercial detergent, (C) Chemical surfactant (SDS), and (D) Distilled water (negative control).

Figure 4. Emulsification index ($E_{24}$) of burnt motor oil with biosurfactants produced by *Rhizopus* strains.

The significant positive values were considered to be above 50% after 24 hours of emulsion according to Kebbouche-Gana et al. (2009); thus, the best emulsification property against burnt motor oil were observed by *R. microsporus* var. *microsporus* UCP 1304 (94.8%), followed by *R. microsporus* var. *chinensis* UCP 1296 (91.7%) and *R. arrhizus* UCP 1607 (82.6%) of emulsification.

All results obtained in this study were higher than the biosurfactants produced by *Aspergillus niger* CF12 (18.5%) and *Rhizopus nigricans* CF3 (21.66%), as described by Lodha et al. (2016).

In addition, the current study suggests that the importance of the screening methods mainly based on primary and secondary assays led to isolation of biosurfactant and bioemulsifying agents using mycelia-free broths, in particular from filamentous fungi. Those assays considered the important properties of higher dispersion oil activity, lower surface tension of the tension active agent, and bioemulsification. According to Uzoigwe et al. (2015), emulsification index test is a suitable screening method for detection of bioemulsifier producing microorganisms.

Most of the surfactants compounds are chemically synthesized. However, the main drawback for the biosurfactants commercialization is associated with non-economical production and is not yet competitive with the synthetics products. The renewable substrates used, especially from industrial wastes as soybean post-frying oil, supplemented with sodium glutamate showed excellent results to biosurfactant and bioemulsifier production at an experimental scale. The agro-industrial waste soybean post-frying oil is considered as the promising substrate for reduction of the cost of production to tenso-active and emulsifier molecules (Andrade Silva et al., 2014; Freitas et al., 2014).

**Conclusion**

In this work, four biosurfactant/bioemulsifier-producing strains were isolated from Caatinga soil of Brazil, namely *R. arrhizus* var. *arrhizus* UCP 1295, *R. microsporus* var. *chinensis* UCP 1296, *R. microsporus* var. *microsporus* UCP 1304 and *Rhizopus* spp. UCP1607, collectively identified as *R. arrhizus*. The experimental result showed
that among the four strains, the best biosurfactant activity was produced in *R. arrhizus* UCP 1607 strain. The biosurfactant produced by *R. arrhizus* UCP 1607 strain had a large hemolytic activity, parafilm drop, oil-spreading diameter, and low surface tension, while the best emulsifying activity was observed using *R. microsporus* var. *microsporus* UCP1304. The screening methods mainly based on surface tension determination have led in many cases to the elimination of bioemulsifying agents. The promising physico-chemical results showed that evaluation of emulsifying activities from mycelia-free broths demonstrated great possibility for production of bioemulsifiers with powerful potential to induce stable emulsion. The better surface active properties with its great effectiveness confirmed the lowering of surface tension and oil dispersion in water by the new strain of *R. arrhizus* UCP 1607. Further studies are under way to scale up growth conditions and to optimize biosurfactant and bioemulsifier productions.

**CONFLICT OF INTERESTS**

The authors have not declared any conflict of interests.

**ACKNOWLEDGEMENTS**

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