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Screening of potential biosurfactant-producing yeasts isolated from the mangroves of the Paranaguá Estuarine Complex (PEC)

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ABSTRACT. Mangroves have been critical to discovering microbial diversity and for their use in biotechnological resources. The Paranaguá Estuarine Complex (PEC) is surrounded by mangroves that are exposed to anthropogenic actions resulting from industrial and seaport activities. The present study evaluated the molecular diversity of mangrove yeast isolates obtained from the PEC. Furthermore, the ability of these microorganisms to degrade oil was researched using the redox indicator 2,6-dichlorophenol indophenol (DCPIP) and dehydrogenase activity assays. Biosurfactant production was screened using the blue agar plate method, drop-collapse test, tilted glass slide test, oil spreading method, and emulsification assay. Internal Transcribed Spacer (ITS) sequencing revealed a great diversity of yeast species in the PEC, including *Candida* sp., *C. parapsilosis, C. tropicalis, Meyerozyma carpophila, M. guilliermondii,* and *Debaryomyces hansenii*. The isolates *D. hansenii* A2M1(1A), *M. carpophila* P2M5(74), and *Candid*a sp. P2M3(74) tested positive for DCPIP and dehydrogenase activity. All isolates showed positive results in the blue agar plate method. The isolates *M. carpophila* A2M3(20), *D. hansenii* A2M3(15), *C. tropicalis* A2C5(2), *C. parapsilosis* A2C5, and *M. carpophila* P2M3(42) indicated activity in the tilted glass slide test. The isolates *C. tropicalis* A2C5(2), *M. carpophila* P2M3(42), and *M. carpophila* P2M5(74) showed activity in the emulsification assay. These findings provide important information about the composition of yeasts in the PEC mangrove, highlighting their potential for application in bioremediation processes of environments contaminated by petroleum.

Keywords: Crude oil degradation; bioremediation; ITS.

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Introduction

Mangroves are coastal ecosystems found in transitional zones between terrestrial and marine environments, typically in tropical and subtropical regions. They serve as habitats and marine nurseries for various animal species (Rog et al., 2017). They are considered favorable sites for essential processes such as nutrient cycling, which is directly related to the activity of microbial communities (Jia et al., 2020). In tropical and subtropical mangroves, microbial communities continuously transform organic agents into sources of nitrogen, phosphorus, and other nutrients (Yang et al., 2017). Additionally, coastal mangroves have been identified as hotspots for discovering microbial diversity and cultivating species for their use in biotechnological resources (Zhu et al., 2017; Al-Hawash et al., 2018).

Brazil harbors about 7% to 9.4% of the world's mangroves and is among the three largest mangrove areas (Hamilton & Casey, 2016). Despite its importance, approximately 35% of mangroves have been destroyed since the 1980s due to anthropogenic activities (Romañach et al., 2018). In Paraná State, south of Brazil, mangroves are located around the Paranaguá Estuarine Complex (PEC), where they are exposed to anthropogenic actions due to industrial and port activities (de Souza Queiroz et al., 2017). Accidents involving oil spillage are considered frequent events and contribute significantly to mangrove degradation (Bhattacharyya et al., 2015; Ferreira & Lacerda, 2016), as these environments tend to accumulate organic contaminants (Zhu et al., 2017).

Petroleum is a complex mixture of aliphatic, alicyclic, and aromatic hydrocarbons, including polycyclic aromatic hydrocarbons (PAHs). Therefore, petroleum is highly harmful to human health because of its carcinogenic, mutagenic, and neurotoxic properties (Marchand et al., 2017). Many authors have highlighted

the ability of several different microorganisms to degrade petroleum hydrocarbons (mainly bacteria, fungi, and yeasts) (Al-Hawash et al., 2018; Marchand et al., 2017). Biosurfactants produced by microorganisms can be employed for the bioremediation of areas contaminated by oils, especially petroleum, as they reduce the surface tension of the water-immiscible substrate, making it more accessible for microbial uptake and metabolism (Sharma et al., 2019; da Silva et al., 2017). Rhamnolipids, a biosurfactant of the glycolipid class produced by microorganisms as bioactive secondary metabolites, show significant biotechnological potential as they can be used in the depollution of oceans and beaches caused by oil spillages. In this line, rhamnolipids are also effective in soil remediation or against environmental pollutants derived from refinery products (e.g., kerosene, gasoline, diesel, benzene, toluene), pesticides, and heavy metals such as cadmium (Sharma et al., 2019).

The present study aimed to verify the molecular diversity of isolated mangrove yeast obtained from the PEC, including an initial evaluation of their bioremediation properties and biosurfactant production. These findings contribute to the exploration of the biotechnological potential of mangrove yeasts for future biotechnological studies and applications.

Material and methods

Fungal isolate collection

Ten yeasts were selected from the culture collection of the Laboratory of Molecular Genetics and Microorganisms (LAGEM, as per its acronym in Portuguese). The isolates were preserved using the Castellani method (Castellani, 1963). These microorganisms were isolated from two different mangroves within the PEC. One mangrove is located in the center of the Palmito State Forest (25° 34.095'S 48° 31.733'W) on the banks of the Itiberê River, and the other, considered anthropized, is located on the banks of the Rio da Vila (25° 33.227'S 48° 31.898'W), an urban perimeter of the Paranaguá municipality, constantly suffering anthropic pressures. The isolates were reactivated in Sabouraud Dextrose Broth (SDB) for seven days at 28 ºC.

Molecular characterization

Genomic DNA extractions were performed using the methodologies of Doyle & Doyle (1987). DNA concentration was estimated using a NanoDrop™ spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA). The Internal Transcribed Spacer (ITS) region was amplified using the primers ITS1 and ITS4 (White et al., 1990). PCR conditions were 5 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 1 min at an adequate annealing temperature, 1 min at 72 °C, 3 min finishing step at 72 °C, and a cool-down step to 4 °C. Amplicons were sequenced using both PCR primers with a BigDye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. Sequences were analyzed on an ABI Prism 3700 DNA Sequencer (Perkin-Elmer, Norwalk, Foster City, CA, USA). BLAST analyses were conducted in GenBank (http://www.ncbi.nlm.nih.gov).

The sequence data obtained were aligned using Muscle (Edgar, 2004) and manually improved using MEGA X (Kumar et al., 2018) when required. Phylogenetic analysis was performed in MEGA X using the maximum likelihood (ML) method, and ModelTest identified the best substitution model through 1,000 bootstrap replicates. Sequences of type strains were used as outgroup terminals (*Coprinopsis cinerea* ATCC MYA-4618), while conspecific sequences were used as ingroup terminals. Sequences derived in this study have been deposited into GenBank. The tree with the highest log-likelihood (-1083.7740) is shown. Initial tree(s) for the heuristic search were obtained automatically using the Neighbor-Joining and BioNJ algorithms on a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with superior log-likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories [+ G, parameter = 0.6541]), and the rate variation model allowed some sites to be evolutionarily invariable ([+ I], 40.6235% sites). The analysis involved 46 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + non-coding, whereas all positions containing gaps and missing data were eliminated. DNA sequences of the identified isolates were deposited in the GenBank database (http://www.ncbi.nlm.nih.gov).

Initial evaluation of a petroleum-degrading carbon source

Crude oil made up of 26% Nigerian oil, 21% Pre-Salt oil, and 53% Campos Basin oil was provided by the company Petrobrás S.A. (Brazil).

Indicator redox 2,6-diclorofenol indofenol (DCPIP)

The indicator redox 2,6-dichlorophenol indophenol (DCPIP) was used to screen potential petroleum-degrading yeasts following a method modified by Hanson et al. (1993). In triplicate, the assay was determined using a 96-well plate containing 190 µL, 185 µL, and 180 µL of Bushnell-Haas (BH) medium, with 0.01% DCPIP added to each well. In addition, crude oil (10 µL, 15 µL, and 20 µL) was added as the sole carbon source, respectively, along with 50 µL of culture inoculum (1.5×108 CFU mL^{-1}). Negative controls contained only BH and DCPIP. The plates were covered and incubated for 48 hours at 32 °C and 120 rpm. The strains that promoted decolorization or color reduction of BH medium supplemented with DCPIP and crude oil in a shorter time were selected for identification and subsequent experiments (Bidoia et al., 2010).

Dehydrogenase activity

Dehydrogenase activity (Braddock & Catterall, 1999) was determined in triplicate using 96-well microplates. Each well contained 140 µL, 130 µL, or 120 µL of culture medium, 150 BH medium with 0.015% triphenyl tetrazolium chloride (TTC) redox indicator, 10 µL, 20 µL, or 30 µL of oil, and 50 µL of microbial suspension (1.5×10^8 CFU·mL^{-1}), totaling 200 µL per well. Negative controls did not include microbial suspension. The plates were covered with aluminum foil and incubated at 32 °C for seven days, with periodic homogenization in a shaker. The evaluation was carried out at intervals of 24, 48, and 168 hours by observing the color of each sample.

Screening for biosurfactant production

Five qualitative screening methods were used to evaluate biosurfactant production and activity by ten yeast isolates. The blue agar plate method was needed to confirm the anionic biosurfactant's glycolipid nature, such as rhamnolipid (Jiang et al., 2020). Three tests were performed to investigate the effect of the produced biosurfactant on surface tension, including the drop-collapse method, the tilted glass slide test, the oil spreading method, and the emulsifying potential as measured by the emulsification index (E24 and E72).

For the tests, the yeast was inoculated in 50 mL of SDB supplemented with 1% extra-virgin olive oil and incubated for three days at 28 °C and 180 rpm. Subsequently, two samples were tested: a) cells suspended in the medium broth and b) cell-free supernatant obtained by centrifuging the culture medium at 1200 rpm for 2 min. All these analyses were performed in duplicate.

Blue agar plate method

For the detection of extracellular anionic surfactants (Siegmund & Wagner, 1991), minimal salt broth medium agar was supplemented with glucose as a carbon source (20 g mL^{-1}), CTAB (0.2 g mL^{-1}), and methylene blue (0.005 g mL^{-1}). The plate was inoculated with disks saturated with yeast culture (pre-grown in SDB containing 1% extra-virgin olive oil) and incubated at 28 °C for seven days. The formation of a dark blue halo zone around the colonies was considered a positive indicator of anionic biosurfactant production.

Drop-collapse method

A total of 100 µL of Lubrax™ 10w40 oil was transferred to the 96-well microplate, and 10 µL of the cell-free culture broth supernatant was spotted at the center of the oil. The drop size was visualized after 1 minute. Equal volumes of CTAB and SDS were used as positive controls. Distilled water was used as the negative control. The same screening was performed with mineral oil. Drop collapse indicated biosurfactant production; a beaded drop was considered a negative outcome (da Silva et al., 2017).

Tilted glass slide

A cell batch of each tested yeast was mixed with NaCl (0.9 %) on the edge of a clean glass slide. The slide was tilted (45º), and the presence of biosurfactant in the sample was confirmed by dripping off the drop (Varjani & Upasani, 2017). Equal volumes of NaCl 0.9% and SDS were used as negative and positive controls, respectively.

Oil spreading method

An oil membrane was formed by adding 10 mL of Lubrax™ 10w40 oil over 50 mL of distilled water dispensed in a Petri dish (10 cm diameter), and 100 µL of the cell-free culture broth was gently added to the

membrane center. After 1 minute, the diameter of the clearing zone around the cell-free culture broth was measured. Equal volumes of CTAB and SDS were used as positive controls, while distilled water served as the negative control. The opening of a halo on the oil drop indicates a positive result (Morikawa et al., 2000).

Emulsification index (E_{24})

Equal volumes (2 mL) of the cells suspended in the medium broth and cell-free supernatant were added to a test tube containing equal amounts of kerosene. The tube was vortexed for 2 minutes at maximum speed and then left to stand for 24 hours at room temperature. The emulsification index (E_{24}) was defined by the following equation:

$E_{24} = (H_{emulsification} / H_{total}) \times 100$

where: E_{24} = emulsification index in 24h; $H_{emulsification}$ = Emulsion height (cm); H_{total} = Total solution height (cm). Equal volumes of distilled water and SDS were used as negative and positive controls, respectively. An emulsification index (E_{24}) above 50% is a positive result for biosurfactant production. The same tube was left to stand for 72 hours (E_{72}) at room temperature to evaluate the stability of the emulsion (Varjani & Upasani, 2016).

Results

Molecular characterization

Partial sequences of the ITS gene from the isolates (Table 1) were aligned with other sequences of 46 recognized species found in the GenBank database. ITS sequencing revealed three distinct genera among the ten isolates*: Candida, Meyerozyma,* and *Debaryomyces* (Figure 1). Four isolates belonged to the genus *Meyerozyma*: isolate P2M3(35) was identified as *M. guilliermondii*, which forms a species complex with the closely related isolates *M. carpophila* A2M3(20), P2M5(74), and P2M3(42). Isolates A2M3(15) and A2M1(1A) were identified as *Debaryomyces hansenii*. The remaining isolates belonged to the genus *Candida*: P1C5 and A2C5 were identified as *C. parapsilosis*, and A2C5(2) as *C. tropicalis*. Isolate P2M3 (74) could not be identified at the species level because it did not cluster with available sequences for the species *C. intermedia* and *C. pseudointermedia* type strains. The non-clustering of these species excluded the possibility that the P2M3 isolate belonged to them, and it was identified as *Candida* sp.

Table 1. Collection details and GenBank accession numbers of isolates included in this study.

Species	Isolate	GenBank accession number[1]
Candida parapsilosis	P1C5(2)	OK037155
Meyerozyma carpophila	A2M3(20)	OK037156
Debaryomyces hansenii	A2M3(15)	OK037157
Debaryomyces hansenii	A2M1(1A)	OK037158
Candida tropicalis	A2C5(2)	OK037159
Candida parapsilosis	A2C5	OK037160
Meyerozyma carpophila	P2M5(74)	OK037161
Meyerozyma carpophila	P2M3(42)	OK037162
Meyerozyma guilliermondii	P2M3(35)	OK037163
Candida sp.	P2M3(74)	OK037164

[1]Internal transcribed spacer (ITS) nuclear ribosomal.

Initial evaluation of petroleum degradation

Six isolates tested positive in the DCPIP assay (Table 2). *D. hansenii* A2M1 (1A), *M. carpophila* P2M5 (74), and *Candida* sp. P2M3(74) also showed positive results for dehydrogenase activity. Additionally, *C. tropicalis* A2C5 (2) only exhibited dehydrogenase activity but did not test positive in the DCPIP assay.

Screening for biosurfactant production

Table 3 presents the results of initial screenings for petroleum degradation and biosurfactant production. All isolates produced dark blue halos around the colony and were considered positive in the blue agar plate method. However, the drop collapse and oil spreading methods demonstrated no activity for 100% of the samples, regardless of whether they used mineral oil or Lubrax™ 10W40 oil substrates with both cells suspended and cell-free aliquots. The isolates *M. carpophila* A2M3(20), *D. hansenii* A2M3(15), *C. tropicalis* A2C5(2), *C. parapsilosis* A2C5, and *M. carpophila* P2M3(42) indicated activity in the tilted glass slide assay.

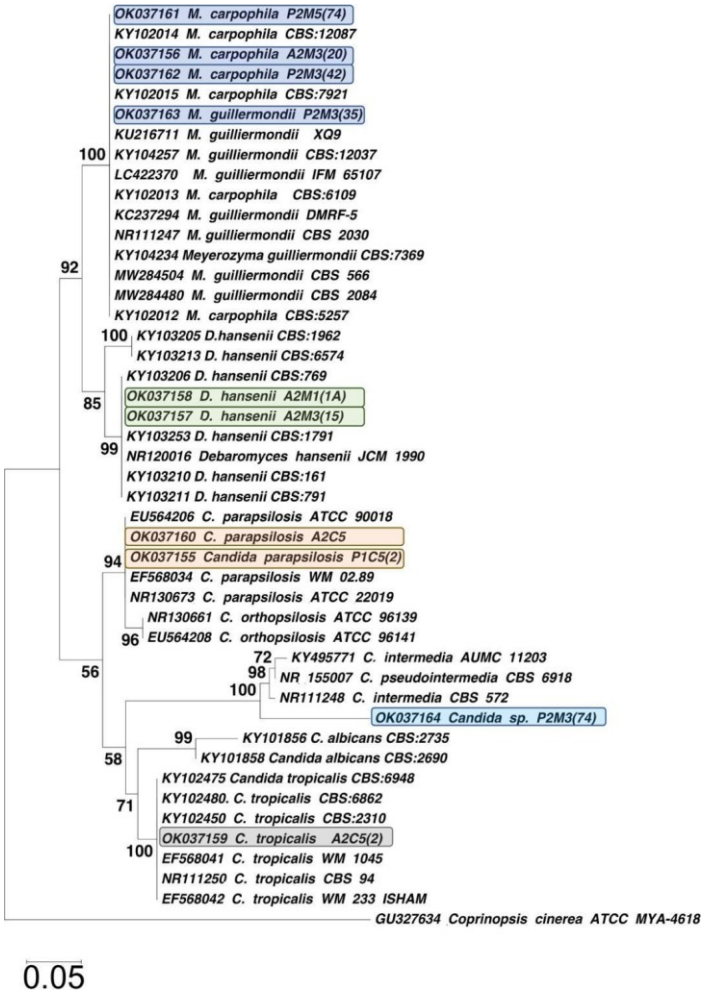

Figure 1. Phylogenetic tree generated from sequencing a partial region of the ITS gene of yeast isolated from mangroves' rDNA, derived from the Maximum Likelihood method and bootstrap test with 1000 replicates. *Coprinopsis cinerea* ATCC MYA-4618 was used as an outgroup.

Table 2. Results for petroleum degradation assays using the redox indicator 2,6-dichlorophenol indophenol (DCPIP) and dehydrogenase activity.

Isolates	DCPIP		Dehydrogenase activity		
	7 days	14 days	24 h	48 h	168 h
C. parapsilosis P1C5(2)	-	-	-	-	-
M. carpophila A2M3(20)	+	+	-	-	-
D. hansenii A2M3(15)	+	+	-	-	-
D. hansenii A2M1(1A)	+	+	+	+	+
C. tropicalis A2C5(2)	-	-	+	+	+
C. parapsilosis A2C5	-	-	-	-	-
M. carpophila P2M5(74)	+	+	+	+	+
M. carpophila P2M3(42)	-	-	-	-	-
M. guilliermondii P2M3(35)	+	+	-	-	-
Candida sp. P2M3(74)	+	+	+	+	+

Table 3. Results for biosurfactant production screenings.

Isolates	BAP	DCM				TG	OS	EA (%)[1]			
		MO		LO				E_{24}		E_{72}	
		CS	CF	CS	CF			CS	CF	CS	CF
C. parapsilosis P1C5(2)	+	-	-	-	-	-	+	60	44	20	44
M. carpophila A2M3(20)	+	-	-	-	-	-	-	40	28	12	20
D. hansenii A2M3(15)	+	-	-	-	-	+	-	64	32	60	24
D. hansenii A2M1(1A)	+	-	-	-	-	-	-	68	0	48	0
C. tropicalis A2C5(2)	+	-	-	-	-	+	+	64	72	64	68
C. parapsilosis A2C5	+	-	-	-	-	-	-	80	32	76	20
M. carpophila P2M5(74)	+	-	-	-	-	-	-	64	80	4	32

M. carpophila P2M3(42)	+	-	-	-	-	-	-	52	80	4	80
M. guilliermondii P2M3(35)	+	-	-	-	-	+	-	48	20	20	0
Candida sp. P2M3(74)	+	-	-	-	-	-	-	20	0	8	0

BAP: Blue agar plate method; DCM: Drop-collapse method; MO: Mineral oil; LO: Lubrax™ 10W40 oil; TG: Tilted glass slide test; OS: Oil spreading method; EA: Emulsification activity. CS: Cells suspended. CF: Cell-free. [1]High activity > 50%.

According to the emulsification activity observed during the first 24 hours of resting stability, the isolated *C. tropicalis* A2C5(2) remained active until 72 hours, demonstrating activity in both cell-suspended broth and cell-free broth cultures. The isolate *M. carpophila* P2M3(42) showed good stability at both 24 and 72 hours for the culture solution with cell-free broth. In comparison, *M. carpophila* P2M5(74) showed activity for the culture solution with cells-suspended broth and the culture solution with cell-free broth only at 24 hours.

Discussion

Our study revealed that the Paranaguá Estuarine Complex hosts a great diversity of yeast species belonging to the genera *Candida*, *Meyerozyma,* and *Debaryomyces*, including *Candida* sp., *C. parapsilosis*, *C. tropicalis*, *M. carpophila*, *M. guilliermondii,* and *D. hansenii*. Bacteria, fungi, and yeasts have been reported to play a significant role in forming residues in mangrove ecosystems (Al-Hawash et al., 2018; Marchand et al., 2017). Fungi, in particular, represent a rich source of structurally unique and diverse bioactive secondary metabolites within the mangrove microbial community, attracting significant attention in biotechnology (Jia et al., 2020). Several studies suggest that mangrove fungi facilitate host adaptation to extreme environments, highlighting their promising character as sources for screening new products (Al-Hawash et al., 2018).

The species diversity observed was confirmed through molecular identification based on ITS sequences. These sequences, along with the 5.8S gene, are part of the CBS-KNAW Fungal Biodiversity Centre's barcode collection, which includes around 9,000 strains and approximately 1,700 yeast species. This collection supports the use of ITS and LSU regions for the identification and classification of fungi and yeasts (Vu et al., 2016). According to the authors, the integration of barcode data with information on antibiotic resistance and the production of industrially or biotechnologically relevant metabolites provides valuable insights for researchers working with yeasts (Vu et al., 2016).

Microorganisms with catabolic activities can be found in polluted sites, speeding up crude oil degradation. Studies involving the degradation of petroleum hydrocarbons by microorganisms have reported that yeasts of the genus *Candida* preferentially degrade aromatic hydrocarbons (Hesham et al., 2009). The yeast *C. tropicalis* has been demonstrated to degrade a wide range of hydrocarbons by completely metabolizing n-alkanes. On the other hand, it was unable to fully degrade aromatic hydrocarbons (phenanthrene, anthracene, fluoranthene, and pyrene) as the sole carbon source (Gargouri et al., 2015).

The synthesis of hydrocarbons by yeasts *D. hansenii* (Kaur et al., 2022), *M. guilliermondii* (Ganapathy et al., 2019), and *C. tropicalis* (Garg & Chatterjee, 2018) has been proven by using glucose or radiolabeled acetate as growth substrates. The synthesis of individual hydrocarbons varied significantly depending on the type of carbon substrate used. Medium-chain alkanes (C16-C19) prevailed in *C. tropicalis.* In contrast, it could not wholly degrade aromatic hydrocarbons (phenanthrene, anthracene, fluoranthene, and pyrene) as the sole carbon source.

The results obtained in this study proved that yeasts from the mangroves of Paranaguá Estuarine Complex can degrade crude oil hydrocarbons. The knowledge of microorganisms and the identification of their ability to biodegrade complex hydrocarbons significantly contribute to improving and applying the bioremediation technique. Besides, it helps maintain and preserve the mangrove. Ten yeast isolates from the genera *Candida*, *Meyerozyma*, and *Debaryomyces* were tested to identify those that can produce biosurfactants based on surfactant activity, emulsifying molecule activity, and biosurfactant class. These tests are promising because they are simple protocols that facilitate the testing, traceability, and quantification of many microorganisms quickly (Varjani & Upasani, 2017).

Among all these methods, the blue agar plate screening method is considered the primary one for biosurfactant producers (Siegmund & Wagner, 1991; Satpute et al., 2010). In our study, all yeasts confirmed the presence of anionic biosurfactant. Studies with yeasts of the genus *Candida* (Garg & Chatterjee, 2018; Nwaguma et al., 2019), *Meyerozyma* (Sharma et al., 2019; Ganapathy et al., 2019; Yurkov et al., 2017), and *Debaryomyces* (Kaur et al., 2022) also showed similar results for initial biosurfactant evaluation.

The drop-collapse method is considered sensitive for rapidly screening rhamnolipid production by various isolates (Satpute et al., 2010). It depends on the principle that a drop of a liquid containing enough

biosurfactant will collapse and spread entirely over the oil surface (Adetunji & Olaniran, 2021). The authors suggest using samples containing cells suspended in the medium broth and cell-free supernatant to assess whether the biosurfactant remains adhered to the yeast wall or is secreted into the culture. The polarity of the microorganism's cell membrane may interfere with the analysis (Satpute et al., 2010).

Moreover, mineral oil and Lubrax™ 10W40 diesel oil screening was performed to observe the interaction between hydrocarbons with different chain sizes and the biosurfactant produced (da Silva et al., 2017; Satpute et al., 2010). In this study, 100% of the samples showed negative results for this screening. However, some studies have reported the possibility of false negatives when the biosurfactant concentration in the analyzed sample is too low or presents a higher emulsifying activity than the surfactant (Satpute et al., 2010).

Due to the need to confirm uncertain results and the possibility of false negatives, some authors suggest using more than one test to reach conclusive results. These may include the tilted glass slide assay, the oil spreading method, and interfacial tension analysis.

The tilted glass slide method works on surface tension analysis and is an effective modified drop-collapse technique. If the surface tension decreases enough to allow the water to flow over the surface, the glass-slide test will be positive (Satpute et al., 2010). In our study, the test detected surface-active compounds for *M. carpophila* A2M3(20), *D. hansenii* A2M2(15), *C. tropicalis* A2C5(2*), C. parapsilosis* A2C5, and *M. carpophila* P2M3(42). *Candida* sp. has been reported to produce biosurfactants that alter the surface tension of glass slides (Garg & Chatterjee, 2018; Nwaguma et al., 2019; Rubio-Ribeaux et al., 2017). The same positive result was reported for organisms of the genus *Meyerozyma* (Ganapathy et al., 2019; Sharma et al*.,* 2019).

The oil spreading method also works on surface tension analysis. It measures the diameter of clear zones caused when a drop of a solution containing biosurfactant is placed on an oil-water surface (Morikawa et al., 2000). The area displaced by a solution containing surfactants is directly proportional to the concentration of the biosurfactants tested. Since all yeasts tested in this study showed negative results, the biosurfactant concentrations produced were likely were below the critical micelle concentration (CMC), which would be insufficient to significantly reduce surface tension.

One explanation for the variation in results between the surface tension analyses may be that the biosurfactant concentration in the sample analyzed was too low, since the reduction in surface tension will only happen if the biosurfactant reaches a specific micellar concentration (Sharma et al*.,* 2019). In addition, environmental and nutritional factors can affect the results. PH, temperature, and oxygen availability are influential factors in the growth of microbes and the quality and quantity of their products (Varjani & Upasani, 2017). Studies show that the structure and production of secondary metabolites depend on microbial growth, medium composition, and the synergistic interaction effect between microorganisms (Ramdass & Rampersad, 2021).

The ability to enhance the solubility and biodegradation of hydrocarbons depends on the structure and quantity of biosurfactants. Meanwhile, the volume and structure of biosurfactants are influenced by growth conditions, including the availability of carbon sources, nutrients in the culture medium (e.g., nitrogen, phosphorus, and iron), temperature, pH, aeration, and other in vitro factors. This could explain the inconclusive results for biosurfactant activity.

Finally, to confirm the production of biosurfactants with emulsifying characteristics, it was observed that among the isolates that tested negative in the drop-collapse and oil spreading methods, *D. hansenii* A2M3(15), *C. tropicalis* A2C5(2), *C. parapsilosis* A2C5, and *M. carpophila* P2M3(42) demonstrated emulsifying capacity (E24) and good emulsification stability (E72). The emulsification assay assumes that if the cell-free culture broth contains biosurfactants, the hydrocarbons present in the test solution will be emulsified. While a direct correlation between surface tension, interfacial tension, and emulsification activity has been used as a screening method, the ability of a biomolecule to form a stable emulsion is not always associated with its capacity to reduce surface tension (Varjani &Upasani, 2017).

The results indicate that isolated *C. tropicalis* A2C5(2) has potential for future environmentally friendly applications. *C. tropicalis* showed potential in biodegrading petroleum oil, some hydrocarbons, and phenol (Al-Dhabaan, 2021). The yeast species *C. tropicalis* is capable of significantly removing crude oil in petroleum-degrading tests, while also producing biosurfactants with great emulsifying ability (Rubio-Ribeaux et al., 2017; Barakat, 2017).

D. hansenii has records of good performance in emulsifying biomolecule production, biosurfactants, and a high potency in petroleum oil biodegradation (Loeto et al., 2021; Kaur et al., 2022), directly related to the

results presented in this study. Although *C. parapsilosis* did not show positive results for crude oil biodegradation in this study, it exhibited good emulsifying activity. *Candida albicans*, *C. lipolytica*, and *C. tropicalis* showed functional groups associated with biosurfactant production like those detected in this study (Garg & Chatterjee, 2018; Mendes da Silva Santos et al., 2021).

The biodegradation of petroleum hydrocarbons by natural populations of microorganisms represents one of the primary mechanisms by which these pollutants can be eliminated from the environment (Lima et al., 2019). This process, known as intrinsic bioremediation, occurs through the activity of microorganisms already present at the contaminated site, without the addition of external agents such as electron donors, acceptors, nutrients, or exogenous microbial strains (Truskewycz et al., 2019).

Conclusion

The Paranaguá Estuarine Complex harbors a great diversity of yeast species belonging to the genera *Candida*, *Meyerozyma*, and *Debaryomyces*, including *Candida sp.*, *C. parapsilosis*, *C. tropicalis*, *M. carpophila*, *M. guilliermondii*, and *D. hansenii*. The yeasts isolated in this study demonstrated potential for crude oil biodegradation and biosurfactant production, indicating their relevance in biotechnological and environmental applications. Since the search and isolation of microorganisms from mangroves were successful in this study, further research should be conducted using qualitative tests to detect biosurfactants, better characterize their properties and optimize their application in bioremediation.

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