Molecular identification of non-conventional yeasts and the screening for α-terpineol assimilation

Identificação molecular de leveduras não convencionais e triagem para assimilação de α-terpineol

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ABSTRACT

The present research aimed to analyze the potential consumption of a monoterpene compound, α-terpineol, by aroma-producing Yeasts. For this purpose, 23 non-conventional yeasts were selected and identified by molecular tools. Subsequently, a screening test was performed to evaluate their tolerance to α-terpineol, by cultivating them in YM broth, at pH 5.0, for 48 h. A mixture of α-terpineol and ethyl alcohol was added to the broth in concentrations ranging from 2.5 to 10.0 µL/mL (intervals of 2.5).

The yeasts that survived any of these concentrations were cultured again, but this time in mineral DP liquid culture medium to assess the consumption of α-terpineol. TLC analysis was conducted to analyze the consumption of α-terpineol. The yeasts studied were identified as Clavispora lusitaniae (n=12), Rhodotorula mucilaginosa (n=8), and Lodderomyces elongisporus (n=3). Furthermore, according to the phylogenetic tree, two of these strains (C. lusitaniae and L. elongisporus) have greater genetic proximity than the yeast R. mucilaginosa. From the 23 yeasts, 6 of them were shown to be tolerant to the concentration of 2.5 µL/mL of α-terpineol. The tolerant strains were identified as C. lusitaniae (n = 1), L. elongisporus (n = 2), and R. mucilaginosa (n = 3). However, the yeast that resisted the highest concentration, R. mucilaginosa, was only 12 hours in all concentrations. It is more likely that the defense mechanism of the yeast was not able to prevent further damage to the membrane as the time in contact with α-terpineol increased. The TLC results showed that the extracts from R. mucilaginosa (CMRP3205) and L. elongisporus (CMRP3192) could be interpreted as potentially promising results of new compounds production by the yeasts.

Keywords: aroma-producing yeasts, molecular identification, non-conventional yeasts, thin layer chromatography.

RESUMO

A presente pesquisa teve como objetivo analisar o consumo potencial de um composto monoterpeno, α-terpineol, por leveduras produtoras de aromas. Para este fim, 23 leveduras não convencionais foram selecionadas e identificadas por ferramentas moleculares. Posteriormente, foi realizado um teste de triagem para avaliar sua tolerância ao α-terpineol, cultivando-os em caldo de YM, a pH 5,0, durante 48 h. Uma mistura de α-terpineol e álcool etílico foi adicionada ao caldo em concentrações que variam de 2,5 a 10,0 µL/mL (intervalos de 2,5). As leveduras que sobreviveram a qualquer uma destas concentrações foram novamente cultivadas, mas desta vez em meio de cultura líquido mineral DP para avaliar o consumo de α-terpineol. A análise TLC foi realizada para analisar o consumo de α-terpineol. As leveduras estudadas foram identificadas como Clavispora lusitaniae (n=12), Rhodotorula mucilaginosa (n=8), e Lodderomyces elongisporus (n=3). Além disso, segundo a árvore filogenética, duas dessas cepas (C. lusitaniae e Lodderomyces elongisporus (n=3). L. elongisporus) têm maior proximidade genética do que a levedura R. mucilaginosa. Das 23 leveduras, 6 delas mostraram ser tolerantes à concentração de 2,5 α/mL de α-terpineol. As cepas tolerantes foram identificadas como C. lusitaniae (n = 1), L. elongisporus (n = 2), e R. mucilaginosa (n = 3). Entretanto, a levedura que resistiu à concentração mais alta, R. mucilaginosa, foi de apenas 12 horas em todas as concentrações. É mais provável que o mecanismo de defesa da levedura não tenha sido capaz de evitar danos adicionais à membrana, pois o tempo em contato com α-terpineol aumentou. Os resultados da TLC mostraram que os extratos de R. mucilaginosa (CMRP3205) e L. elongisporus (CMRP3192) poderiam ser interpretados como resultados potencialmente promissores da produção de novos compostos pelas leveduras.

Palavras-chave: leveduras produtoras de aroma, identificação molecular, leveduras não-convencionais, cromatografia de camada fina.

1 INTRODUCTION

In general, Biotechnology can be defined as the study of microscopic or macroscopic living beings aiming for their manipulation and insertion in different processes, to obtain products with some added value, which is known as biotechnological products (Brasil, 2019). Since ancient times, biotechnological processes have been widely used in food and beverage production (Gusmão; Silva; Medeiros, 2017). Thus, much has been studied about the microorganisms responsible for improving the organoleptic and technological characteristics of food and beverages. Including those related to the aroma of the products, as these properties greatly influence consumers' acceptance of the product (Bolha et al, 2020). In this context, seeking to understand the transformation process and production of different biomolecules by these microorganisms to insert them into industrial processes (Kręgiel; Pawlikowska & Antolak, 2017). Non-conventional yeasts are characterized by being able to use different metabolic routes, which provide the production of relevant biomolecules, thus, highlighting their biotechnological potential (Flores et al. 2000, Monteiro de Oliveira et al. 2021).

Thus, the compounds produced by microbial synthesis are considered natural products, meeting the growing market demand for this category of products. For this reason, the amount of research whose main object of study is non-conventional yeasts has grown significantly in the last decades (Berger, 2009). These yeasts are already used in numerous processes, such as in the manufacture of food and food additives (Binati et al., 2021), beverages (Ruiz et al., 2019), drugs, and the synthesis of other biochemical compounds (Kręgiel; Pawlikowska; Antolak, 2017). Therefore, the elucidation of the substrate by microorganisms for the formation of value-added biomolecules is essential for the application of yeasts in industrial processes. The main objective of this work was to analyze the potential consumption of a monoterpene compound, α-terpineol, by non-conventional yeasts.

2 MATERIAL AND METHODS

2.1 YEASTS STRAINS

For this study, we selected a total of 23 non-conventional yeasts (Table 1) with capacity for bioflavors production. The strains were previously isolated by Pietrowski (2018) and are deposited at the Coleção Microbiológica da Rede Paranaense (Microbiological Collections of Paraná Network) collection center of the Taxonline Network. The selected microorganisms were cultured on Sabouraud's Glucose Agar

(glucose 10 g.L^{-1} and incubated at 30 °C for 48h. Stock cultures were maintained frozen (-20°C) in tubes with glycerol 30%.

Table 1 - Selected strains for the tolerance and consumption studies of α-terpineol.

Species	CMRP Number	GenBank accession number
Clavispora lusitaniae	CMRP3160, CMRP3163 CMRP3165, CMRP3167 CMRP3169, CMRP3170 CMRP3171, CMRP3172 CMRP3175, CMRP3177 CMRP3180, CMRP3189	In progress
Lodderomyces elongisporus	CMRP3181, CMRP3184, CMRP3192	In progress
Rhodotorula mucilaginosa	CMRP3195, CMRP3197, CMRP3198, CMRP3199, CMRP3202, CMRP3203, CMRP3204, CMRP3205	In progress

3 MOLECULAR IDENTIFICATION

The identification using molecular techniques was based on sequencing of the ITS rDNA region. DNA extraction and amplification were performed as described by Vicente et al. 2008. Primers used for amplification were LS266 (5´GCATTCCCAAACAACTCGACTC 3´) (de Hoog and Gerrits van den Ende 1998) and V9G (5´ TTACGTCCCTGCCCTTTGTA 3´) (Masclaux et al. 1995) and for sequencing ITS1 GCATTCCCAAACAACTCGACTC) and ITS4 (TCCTCCGCTTATTGATATGC) (White et al. 1990). The sequencing was performed by Myleus Biotechnology Company (www.myleus.com) and the sequences were adjusted using the BioEdit software. Comparisons of the ITS1-5.8S-ITS2 region were performed using the GenBank nucleotide using the BlastN tool analysis (Basic Local Alignment Search Tool). The ITS region was used to reconstruct the phylogeny to confirm the identification of the isolates which shows a similarity of less than 100% with the sequences deposited in the database. Sequences were aligned and manually corrected using the program MEGAX (Kumar et al., 2018). Phylogenetic relationships were estimated using the maximum likelihood method and *Schizosaccharomyces pombe* (CBS 1062) and *Naohidea sebacea* (CBS 8477) were used as the outgroup for comparison.

4 EXPERIMENTAL CONDITIONS

Before the tests with monoterpenes, we established the best experimental growth condition for pH and alcohol tolerance for these yeasts. The initial inoculum suspension was controlled at (10^6 cells/mL). Tolerance of pH was determined using the growth

medium YM (peptone 5 $g.L^{-1}$; glucose 10 $g.L^{-1}$, malt extract 3 $g.L^{-1}$; Yeast extract 3 $g.L^{-1}$) and the pH values tested were pH 5.0, 7.5, and 8.0. The viable cells were evaluated and measured by using a Neubauer chamber. To assess alcohol tolerance, the YPD medium was supplemented with ethanol at concentrations from 0, 12, 33, 19, 25, 66 e 32,33 % (v/v) and the biomass was measured by UV-Vis spectrophotometer. For both tests, the cultures were incubated at 200 rpm at 30°C in the incubator for 48h.

5 SCREENING TEST FOR TOLERANCE TO A-TERPINEOL

The methodology was adapted from Oliveira-Felipe (2015). Yeast colonies (n=23) grown on Sabouraud's Glucose Agar (SGA) were subcultured into tubes with YM broth at pH 5. The tubes were incubated at 30 °C for 24 h. After incubation, cultures were diluted using a McFarland scale of 0.5 and verified by counting in a Neubauer Chamber. Screening tests were performed in a 1,5 mL microtube with 1mL of the diluted culture of which tested strain and supplemented with the α-terpineol (Sigma Aldrich) in different concentrations. The α-terpineol was mixed with an equal part of absolute ethanol, which was filtered in a 0.22 µM Millipore membrane, for better dilution of the terpenic compound in the liquid culture medium. Two positive controls were adopted: (1) a YM broth with glucose (10 g/ as the carbon source; (2) a YM broth with 5 and 10**%** (v/v) alcohol, to confirm the alcohol is not a growth-limiting factor. Negative control was also adopted with the sterilized medium. The tubes were incubated in a thermoblock with agitation (VHD/model B1-AQ/AG-100) at 30 °C under agitation at 700 rpm for 48 h. After 24 and 48 h, the cultures were homogenized and 100 µL were transferred to plates with YM solid medium (1.7% bacteriological agar). We also proceed with this step for the strains that showed growth during the first hours. We performed the cultivation in YM solid medium after 6 h, 12 h, 18 h, and 24 h of contact with α-terpineol. The plates were incubated at 30 °C for up to three days.

6 EVALUATION OF A-TERPINEOL ASSIMILATION

Assimilation of α-terpineol as the sole carbon source was tested for six selected strains based on the screening test (CMRP 3169, CMRP 3181, CMRP 3192, CMRP 3198, CMRP 3204, and CMRP 3205), adapted from Oliveira-Felipe (2015). Experiments were performed in 1,5 mL microtubes. Each microtube contained 1mL of liquid DP mineral medium (NH_4Cl 1 $g.L^{-1}$, K_2HPO_4 0.5 g. L^{-1}, $MgSO_4.7H_2O$ 20 mg. L^{-1}) and 2.5µL/mL of α-terpineol and incubated in a thermoblock with agitation (VHD/model B1-AQ/AG-100)

at 30 °C under agitation at 700 rpm for 48h. Aliquots of supernatant culture were collected every 12 h and frozen until extraction. Positive controls were also used, that is, the yeast culture without α-terpineol, and negative control, prepared only with the sterilized culture medium. As positive controls, the yeast culture without α-terpineol was used, and the negative control was prepared only with the sterilized culture medium.

7 EXTRACTION AND THIN-LAYER CHROMATOGRAPHY ANALYSIS (TLC)

Extraction was performed for all samples, including controls as performed by Llorens-Molina et al. (2017). The preparation of culture supernatants was undertaken by centrifugation at 13,000 rpm for 5 minutes. Then, the supernatants were extracted with absolute ethanol followed by centrifugation at 13,000 rpm for 3 min. Finally, 200 µL of dichloromethane was added and centrifuged again for 3 min. After centrifugation, the phases were separated. The aqueous/ethanolic phase was discarded and the dichloromethane phase was collected in an amber vial and frozen until analysis by thin-layer chromatography (TLC). TLC was performed according to Wagner and Bladt, 2009, using Macherey-Nagel silica gel plates (Dueren, Germany) with UV fluorescence. Plates were identified then incubated at 100ºC for 1h, to activate. Once activated, 10 µL of each sample were applied to each spot. Chromatographic plates after spot sample applications were placed in a vat for saturation with a mixture of toluene and ethyl acetate (93:7) as the mobile phase. The distance was 17 cm from the sample application point. Then, the mobile phase has been evaporated. TLC results were checked with UV light (λ = 365nm). The spots of separated compounds were detected with anise aldehyde solution (p-anisaldehyde 5mL, acetic acid 50 mL, and sulfuric acid 1mL) (Merck Chemical Works, 1971). The plates were heated to 105°C until the maximum spot's visualization. The size, color, and intensity of the spots were observed, and the distance was measured to Retention Factor (RF) calculation. The retention factor was calculated by dividing the distance from the baseline to the solvent front by the distance from the baseline to each sample spot.

8 RESULTS

Yeasts strains were identified on basis of their rDNA ITS sequences. Comparisons of sequence data to the databases led to an assignment to three different genera belonging to *Clavispora*, *Rhodotorula*, and *Lodderomyces*. The yeast species were identified as *Clavispora lusitaniae* (n=12), *Rhodotorula mucilaginosa* (n=8), and three

strains were identified as *Lodderomyces elongisporus*. To confirm the phylogenetic position of some strains (CMRP3189, CMRP3177, CMRP3170, CMRP3192, CMRP3198, CMRP3199, CMRP3203, and CMRP3204) that did not match 100% in BlastN comparison, phylogenetic trees were constructed. Reference sequences from the CBS collection (Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands) were included in the analysis. The phylogenetic trees showed (Figure 1 and 2) that *C. lusitaniae* and *L. elongisporus* have greater genetic proximity than the *R. mucilaginosa* analysis.

Figure 1 - Phylogenetic tree constructed with maximum likelihood, with 500 bootstrap replications, the best evolutionary model was Tamura-Nei with gamma variance and partial deletion. The species *Schizosaccharomyces pombe* (CBS 1062) was used as an outgroup.

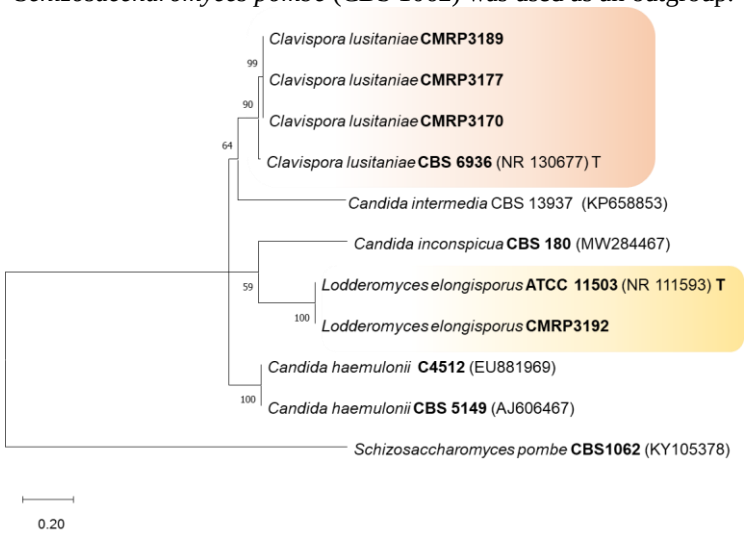
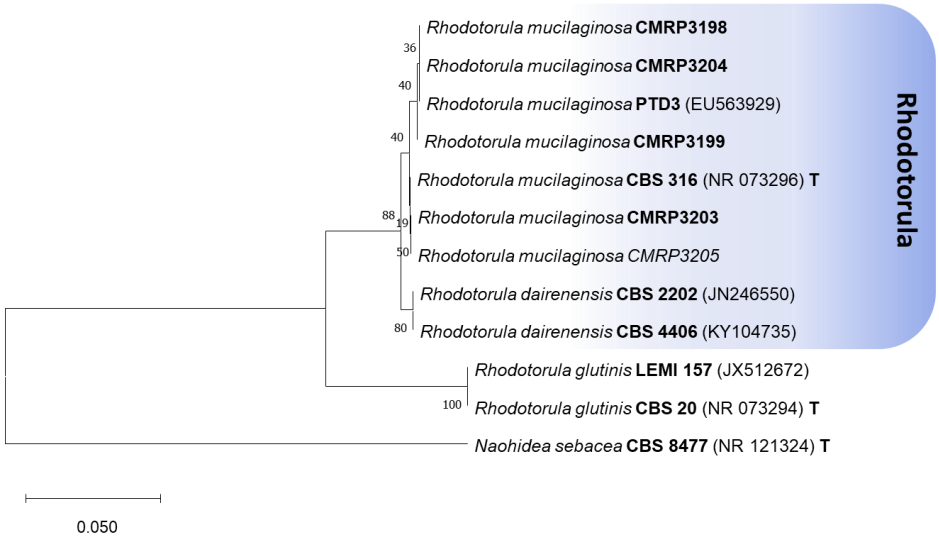


Figure 2 - Phylogenetic tree constructed with Neighbor-Joining, with 500 bootstrap replications, the best evolutionary model was Tamura 3-parameter with gamma distribution and partial deletion. The species *Naohidea sebacea* (CBS 8477) was used as an outgroup. T=type strain



According to the pH tests performed, the optimal pH for microorganisms' growth was pH 5.0. At this pH, greater development of microbial cells was observed in the Neubauer Chamber. Therefore, pH 5.0 was used as a standard in the culture broth for all 23 strains.

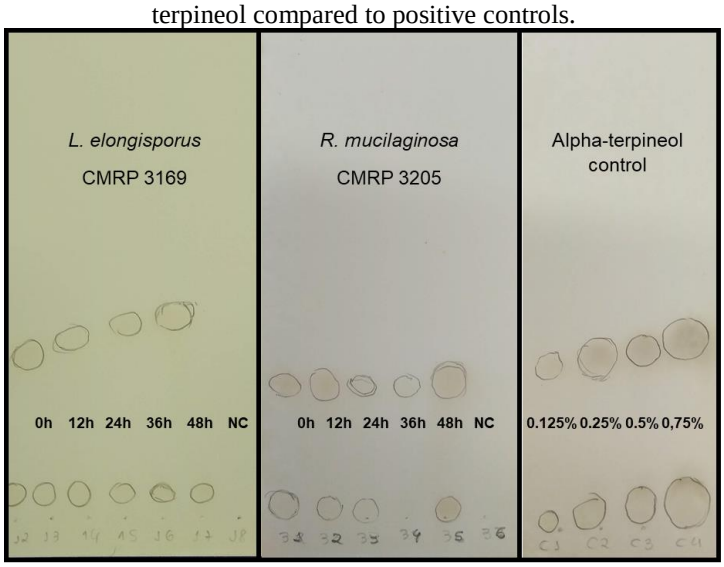
Six strains showed tolerance to α-terpineol from the 23 yeasts screened for α-terpineol at 2.5 µL/mL concentration. One of which also showed tolerance to the 10.0 µL/mL concentration for 12 hours. The tolerant strains were identified as *C. lusitaniae* (CMRP 3169), *L. elongisporus* (CMRP 3181 and CMRP 3192), and *R. mucilaginosa* (CMRP 3198, CMRP 3204, and CMRP 3205). *R. mucilaginosa (*CMRP 3205) resisted in all concentrations however only for 12 hours. With the 6 tolerant strains, we proceeded to evaluate the consumption capacity of this compound as only carbon source and energy. The TLC profiling of *L. elongisporus* (CMRP 3169) and *R. mucilaginosa* (CMRP 3205) are described in Table 2. Images of thin-layer chromatography plates (TLC) were grouped in figure 3.

Table 2 – TLC results for *L. elongisporus* (CMRP 3169) and *R. mucilaginosa* (CMRP 3205).

Species	**Code in TLC plate**	**Incubation time (h)**	**RF**
L. elongisporus CMRP 3169	13	0	0,0235 | 0,2412
	14	12	0,0235 | 0,2705
	15	24	0,0235 | 0,2882
	16	36	0,0235 | 0,2941
	17	48	0,0235
	18	Negative control	-
R. mucilaginosa CMRP 3205	31	0	0,0059 | 0,1941
	32	12	0,0059 | 0,1882
	33	24	0,0059 | 0,1941
	34	36	0,1941
	35	48	0,0059 | 0,1882
	36	Negative control	-
Control results			
Identification	**Code in TLC plate**	**A-terpineol Concentration**	**RF**
Control 1	C1	0.125 %	0,125 0,0059 | 0,2412
Control 2	C2	0.25 %	0,25 0,0059 | 0,2412
Control 3	C3	0.5 %	0,5 0,0059 | 0,2588
Control 4	C4	0.75 %	0,75 0,0059 | 0,2705

Regarding, the *L. elongisporus* CMRP 3169 sample, visibly showed spots that moved more towards the less polar direction as the cultivation time increased, which was evidenced by the RF values which gradually increased from 0.24 to 0.29. In the sample *R. mucilaginosa* (CMRP 3205), the RF values did not show a very significant variation and remained within the controls reference values (C3 and C4).

Figure 3 – TLC plate with results of CMRP 3169 and CMRP 3205 strains from yeast culture with α-terpineol compared to positive controls.



9 DISCUSSION

To contribute to the molecular identification and verify the phylogenetic relationship between the studied strains, phylogenetic trees (Figures 1 and 2) were constructed for the strains which presented an accuracy of less than 100% in comparison with the sequences deposited in the NCBI database. The phylogenetic trees showed that *C. lusitaniae* and *L. elongisporus* have greater genetic proximity than the *R. mucilaginosa*. *Clavispora lusitaniae* is known as a non-conventional yeast, haploid, opportunistic, and belonging to the Ascomycete group (Holzschu et al., 1979). Considered an opportunistic pathogen in humans, responsible for colonizing and infecting humans, and causing disseminated infection, including septicemia and pyelonephritis (Kidd et al., 2016). However, they are also widely found in the environment and have been isolated from water, soil, and plants (Durrens et al., 2017). On the one hand, we have the opportunistic characteristic of this human pathogen. On the other hand, we observe research on the technological potential of this species. In a study that investigated the enzyme production by *C. lusitaniae*, the production of α-L-rhamnosidase was verified, indicating a possible application in the quality improvement of beverages or in the deacidification of citrus juices (Singh; Sahota; Singh, 2015).

Phylogenetically close to *C. lusitaniae*, strains of *Lodderomyces elongisporus* were also identified in this work. This microorganism was associated with blood infections for the first time in 2007, due to the morphological similarity, it was believed to be *Candida parapsilosis* (Lockhart et al., 2007). Due to the morphological similarity

between *L. elongisporus* and other species, molecular identification is the most accurate way to perform correct identification.

As for the biotechnological potential of *L. elongisporus* strains, we can mention their presence and contribution to the final aroma in beverages, which have already been highlighted during the wine fermentation process, composing the initial fermentative microbiota (Ruiz et al., 2019). The authors also highlighted the oenological potential of the species, mainly due to the ability to develop under vinification conditions, such as in high concentrations of ethanol, in addition to the impact caused in the final product, which obtained high concentrations of 3-methyl1-butanol and 2 -phenyl ethanol, and a large amount of protein.

Finally, regarding the identified species *Rhodotorula mucilaginosa*, the species belonging to the Sporidiobolaceae family, and are characterized as pigmented yeasts (Fell, 2000). From the 37 species belonging to the genus, 3 have already been related to infections in humans, namely *R. mucilaginosa*, *R. minuta*, and *R. glutinis* (Biswas et al., 2001). Even though it is also related to cases of infections in humans, this species has biotechnological properties that are considered valuable and for this reason, it is extensively studied (Arendrup, 2014). *Rhodotorula mucilaginosa* has been found in several fermentation processes of different fermented beverages such as Chicha, a traditional drink of the indigenous people from Brazil made by artisanal corn fermentation (Resende et al., 2018); matsoni fermented milk, a traditional food product in Georgia and Armenia (Bokulich et al., 2015), and found associated with *C. lusitaniae*, in the preparation of strong-flavored liqueur from the Yibin region of China (You et al., 2016). This liqueur is made with five types of steamed grains, containing sorghum, glutinous rice, rice, wheat, and corn (You et al., 2016).

Regarding the tolerance studies, among the selected strains, seventeen were not able to develop in the presence of a minimum concentration of 2.5 µL/mL of α-terpineol. The yeast's death may have occurred because they were affected by α-terpineol once the compound had been recognized in several studies with antimicrobial properties (Jing et al. 2015, Li et al. 2015, An et al. 2019). In a study conducted by Jing et al. 2015 with the filamentous fungus *Penicillium digitatum*, the monoterpene compound proved to be more harmful. The minimum inhibitory concentration for this strain was 2.00 µL/mL. The authors concluded that α-terpineol caused the inhibition of fungal growth due to the damage caused in *P. digitatum* cell wall synthesis, which prevented the reconstruction and led to rupture. Another study from Li et al. (2015) evaluated the antibacterial effect

of α-terpineol from *Cinnamomum longepaniculatum* (Gamble) N. Chao leaf essential oils against *Escherichia coli* (CMCC (B) 44102). The study has shown a minimal inhibitory concentration value of α-terpineol was 0.78μL/mL. Park et al. (2009) studied the α-terpineol effect when applied to filamentous fungi *Trichophyton mentagrophytes* and observed the fungus hyphae were morphologically affected when a concentration of 1 mg/mL of α-terpineol was applied. All the studies presented a minimum inhibitory concentration lower than the minimum concentration (2.5 μL/mL) tested in the present study.

From the 6 tolerant strains, only two *L. elongisporus* (CMRP 3169) and *R. mucilaginosa* (CMRP 3205) showed potentially promising results of new compound production. The results of this study led us to conclude that the strains *L. elongisporus* (CMRP 3169) and *R. mucilaginosa* (CMRP 3205) has a significant potential to produce new compounds. The strains also showed interesting results in biomass production during cultivation and visible points on the chromatographic plate. Further research is required to better investigate the production of the new compound. For future analysis, it is proposed the use of auxiliary techniques, such as gas chromatography coupled to a mass spectrometer for greater sensitivity in the detection of these compounds.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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