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MICROBIOLOGY

Extracellular hydrolytic enzymes produced by yeasts from Antarctic lichens

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Abstract: In the Antarctic environment, yeasts are versatile eukaryotes that have shown wide dispersion in different substrates, producing active enzymes in extreme conditions, but their relevance in biotechnological applications is largely unknown. The aim of this study was to evaluate the production of extracellular hydrolases by yeasts isolated from Antarctic lichens and molecularly identify these isolates. From a total of 144 isolates on the screening, 109 (76%) produced at least one of the hydrolases tested, with most activities for proteases 59 (41%), cellulases 58 (40%), esterases 57 (39%), lipases 29 (20%), amylases 23 (16%) and pectinases 20 (14%). Among these isolates, 76 were identified, most belonged to the phylum Basidiomycota (n=73) with the dominance of *Vishniacozyma victoriae* (n=27), *Cystobasidium alpinum* (n=3), *Mrakia niccombsii* (n=3), *Cystobasidium laryngis* (n=2), *Bannozyma yamatoana* (n=2), *Holtermanniella nyarrowii* (n=2), and *Glaciozyma martinii* (n=2). This study is the first one reporting extracellular enzyme production by yeasts isolated from thallus of the species of Antarctic lichens *Lecania brialmontii, Polycauliona candelaria*, *Usnea capillacea*, *Cladonia metacorallifera*, and *Polycauliona regalis*. With these data, it's possible to confirm lichens as a source of hydrolase-producing yeasts, reinforcing the potential of these microorganisms in bioprospecting studies of catalytic molecules from polar regions that may be useful in promising biotechnological applications.

Key words: Bioprospection, cellulase, fungi, lichensphere, *Vishniacozyma*.

INTRODUCTION

Antarctica is a remote continent located in the southern portion of the planet with an extension of approximately 14 million km² and few areas free of permanent ice. It is a habitat marked by low temperatures, strong winds, freeze-thaw cycles, and high ultraviolet radiation (Shivaji & Prasad 2009, Convey 2011). This extreme environment is characterized by severe climatic conditions and challenging biological dispersion that can accommodate particular groups of microorganisms, including yeasts. In Antarctica, investigations of the diversity of these eukaryotes are relatively scarce compared to other microorganisms and have been increasing in recent years (Buzzini et al. 2018, Rosa et al. 2019).

Genetic inferences have shown a high prevalence of yeast representatives of the genera *Aureobasidium*, *Cystobasidium*, *Debaryomyces*, *Glaciozyma*, *Leucoporidium*, *Mrakia*, *Rhodotorula* and *Vishniacozyma* in Antarctica and others cold regions (Ruisi et al. 2007, Buzzini et al. 2018). Yeasts species belonged to these genera and others were found in different

Antarctic substrates, especially in soils (Gomes et al. 2018), mosses (Ferreira et al. 2019), natives plants (Santiago et al. 2017), macroalgae (Duarte et al. 2016), snow (de Menezes et al. 2019), lakes, sediments and in seawater samples (Wentzel et al. 2019). In addition, some studies have reported these microorganisms in lichens, until recently one of the least explored substrates for the presence of yeasts (Duarte et al. 2013, 2016, Santiago et al. 2015).

Lichens are the dominant component of the Antarctic terrestrial ecosystem, widely distributed in the different islands. They are forms of life resulting from symbiotic interaction between filamentous fungi and a photobiont partner (algae or cyanobacteria), although, studies indicate other microorganisms involved in this association and the term lichensphere was introduced by Santiago et al. (2015) to represents the thallus of lichens as a natural microhabitat for refuge and dispersion of non-lichenic microorganisms, such as yeast. The latter strategically take shelter in the lichenic structure and can play functional roles in the relationships, particularly in the sharing of metabolites, that take place in lichens (Santiago et al. 2015, Grube & Wedin 2016, Pankratov et al. 2017, Carvalho et al. 2019). It is advantageous for yeasts to colonize these niches and establish associations as a survival strategy in this extreme environment (Santiago et al. 2015). It is also known that yeasts from Antarctica have adaptations to different selective pressures, with a high proportion of unsaturated fatty acids in the cell membrane, synthesis of cryoprotective compounds, as well as synthesis of enzymes active at low temperatures, which allows them to efficiently use the few nutritional sources available (Buzzini & Margesin 2014).

Exoenzymes are enzymes synthesized and secreted extracellularly and are part of the yeasts nutrition strategy. Exoenzymes production is intrinsically related to the ability of microorganisms to use different macromolecules as energy sources, allowing them to spread across habitats. As the metabolism of yeasts from cold habitats is carried out by exoenzymes active in these thermal conditions (Duarte et al. 2013, 2018, 2021), this ability is useful for survival under the oligotrophy and perennial low temperatures of the Antarctic environment (Buzzini & Margesin 2014).

From molecular aspects, cold-adapted enzymes are more flexible due to the occurrence of more alpha-helices in the secondary conformation, amino acids with simpler side chains, and a lower number/intensity of intramolecular interactions. These properties confer to these enzymes catalytic efficiency and thermal stability in the cold (Pearce 2012, Santiago et al. 2016, Rafiq et al. 2019). Many Antarctic yeasts have been reported to produce a variety of cold-active enzymes, including proteases, amylases, cellulases, glucosidases, lipases, laccases, pectinases, tannases, and xylanases (Duarte et al. 2018). As the adaptations they exhibit are differentiated, and that knowledge on these enzymes are scarce, the study of such biomolecules can bring to light new applications in different biotechnological processes (Martorell et al. 2019). In this context, the aim of this study was to evaluate the production of extracellular hydrolases by yeasts isolated from lichens collected in South Shetland Islands and the Antarctic Peninsula, as well as to taxonomically identify these isolates using molecular tools.

MATERIALS AND METHODS

Sampling of lichens

Twenty-three samples of lichens were collected in the South Shetland Islands (maritime Antarctic) and Antarctic Peninsula during two Brazilian

expeditions: OPERANTAR XXXV (2015/2016) and XXXVI (2016/2017). The photographic field record of the lichens *in loco* was obtained together with GPS data record of the sampling location (Figures 1 and 2). Samples of lichens were packed in distinctly sterile zip-lock bags and stored frozen for further analysis at the Federal University of Alagoas – Campus Arapiraca, Brazil.

Morphological identification of lichens

Lichens were identified according to the taxonomic identification key proposed by Øvstedal & Smith (2001) based on morphological characters. The author citations for the species were provided by the Index Fungorum database (http://www.indexfungorum.org).

Isolation and purification of yeasts

Lichen samples were homogenized in saline solution (0.85%), diluted and plated in Petri dishes using two culture media: **a.** Yeast Malt Agar in $g.L^{-1}$: (yeast extract 3, malt extract 3, peptone 5, glucose 10 and agar 15), and **b.** Sabouraud Dextrose Broth (Kasvi, Brazil) diluted 10 times and was added agar (15 $g.L^{-1}$), both supplemented with amoxicillin (Eurofarma, Brazil, 500 $mg.L^{-1}$) and chloramphenicol (Inlab, Brazil, 100 $\mu g.mL^{-1}$). Inoculated Petri dishes were incubated at 8.0 ±

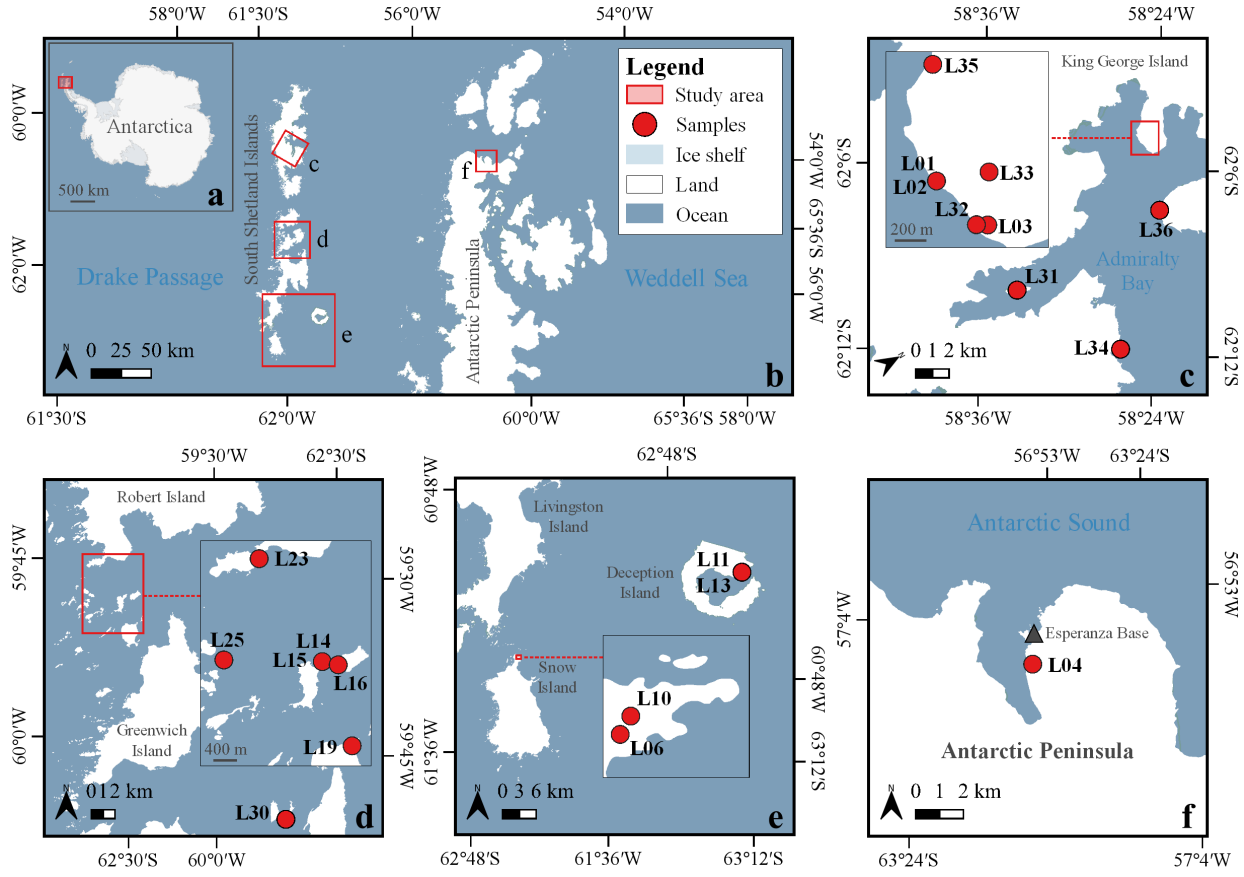

Figure 1. Map of lichen collection sites. a. Overview of the location map. b. Location of South Shetlands Islands and Antarctica Peninsula. c-f. Zoomed-in details of the sampling sites. *The map was generated using QGIS software (version 3.14.15; https://www.QGIS.org) and the SCAR Antarctic Digital Database (ADD version 7.0; http://www.add.scar.org). ** *Cladonia metacorallifera* (L30); *Lecania brialmontii* (L19); *Mastodia tessellata* (L14); *Polycauliona regalis* (L15); *Polycauliona candelaria* (L4, L6, L13); *Rhizocarpon geographicum* (L23); *Sphaerophorus globosus* (L10); *Umbilicaria decussata* (L16); *Usnea antarctica* (L36); *Usnea aurantiacoatra* (L1, L2, L3, L25, L31, L33, L34, L35); *Usnea capillacea* (L11).

2.0 ºC, and growth monitored for 60 days. Yeast isolates obtained were further plated into new culture media for purity confirmation. Thus, purified yeast isolates were cryopreserved at -80.0 °C in 20% glycerol solution.

Extracellular hydrolytic enzyme screening

Enzyme screening was performed as described by Martorell et al. (2017), with the evaluation of the production of six hydrolases enzymes: amylase, cellulase, esterase, lipase, pectinase, and protease. Based on the specificity of each enzyme, yeast isolates were cultured in a solid YMA culture medium, diluted 10 times, with addition of inducing substrates, standardized inoculum at 10^7 cells.mL^{-1} (by spectrophotometry) and incubated for 7 days at 15.0 ºC. Although the yeasts were isolated at 8.0 ºC, it was decided to carry out the screening at 15.0 ºC, as this is the most reported production temperature with cold-adapted enzymes (Martorell et al. 2017).

For amylase screening, the YMA culture medium was supplemented with starch (10 g.L^{-1}), and the activity halo was confirmed with the addition of 1% iodine solution to the plates. For cellulase, carboxymethylcellulose (5 g.L^{-1}) was added to the culture medium and the orange hydrolysis halo revealed by using Congo red solution (1 g.L^{-1}) and NaCl (1M) during 15 minutes. For lipase, olive oil (4%) was added to the growth medium as an inducer together with Rhodamine B dye (0.01%), and UV light indicated the yellowish fluorescent halos. For protease, the medium was added with skimmed milk

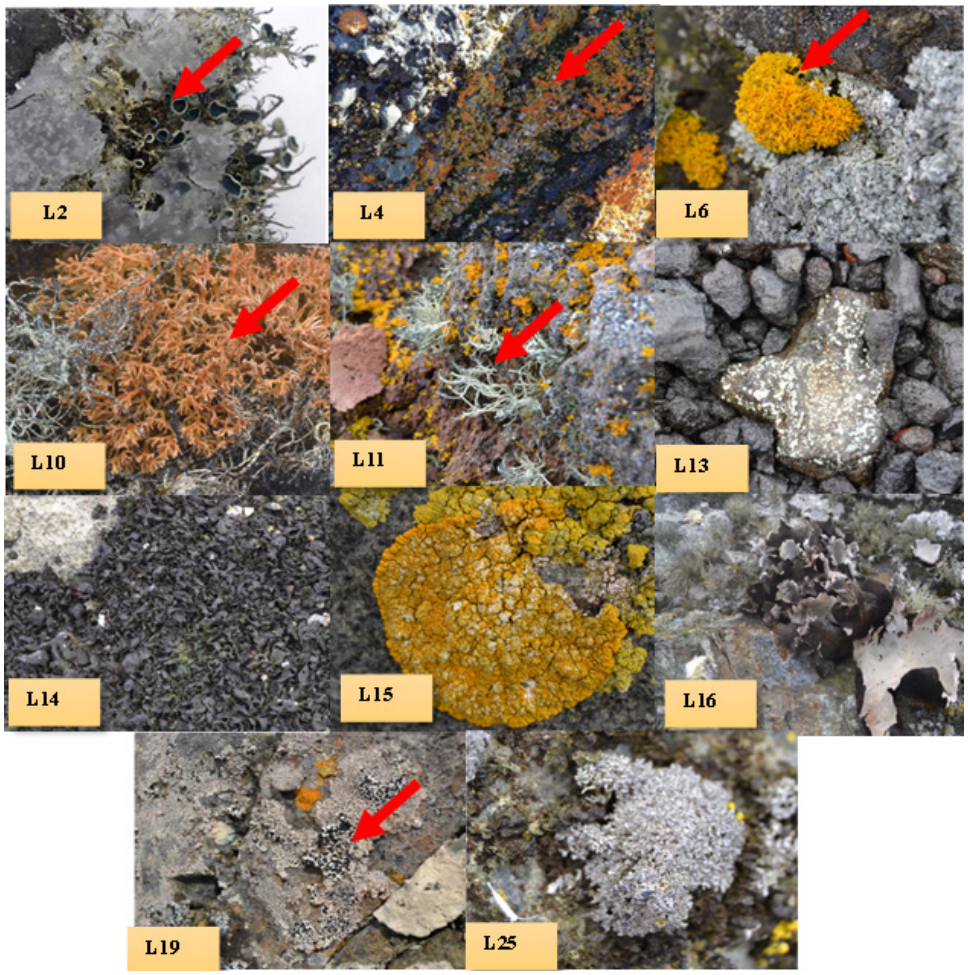

Figure 2. Images illustrative of Antarctic lichens (*in loco*) used in this study for isolation of yeast. * L2. *Usnea aurantiacoatra* (King George Island). L4. *Polycauliona candelaria* (Antarctic Peninsula). L6. *Polycauliona candelaria* (Snow Island). L10. *Sphaerophorus globosus* (Snow Island). L11. *Usnea capillacea* (Deception Island). L13. *Polycauliona candelaria* (Deception Island). L14. *Mastodia tessellata* (Barrientos Island). L15. *Polycauliona regalis* (Barrientos Island). L16. *Umbilicaria decussata* (Cecilia Island). L19. *Lecania brialmontii* (Dee Island). L25. *Usnea aurantiacoatra* (Bilyana Island).

powder (2%) and the clear halos around the colony indicated extracellular activity. While for pectinase evaluation, pectin (10 $g.L^{-1}$) was added as substrate, and cetyltrimethylammonium bromide solution (10 $g.L^{-1}$) was used to confirm the enzymatic activity. For the analysis of esterase, tween 80 (10 $g.L^{-1}$), peptone (10 $g.L^{-1}$), calcium chloride (4 $g.L^{-1}$), and sodium chloride (5 $g.L^{-1}$) were used, with the clear halo indicative of enzymatic activity, visualized by $CaCl_2$ precipitation. Thus, Enzymatic Index (IE), corresponding to the ratio between the value of the hydrolysis halo by the size of the colony, was calculated in order to confirm those isolates with greater extracellular enzymatic activity (Equation 1). All experiments were performed in duplicate.

$$Enzymatic\ Index = \frac{Halo\ diameter\ (mm)}{Colony\ diameter\ (mm)} \quad (1)$$

Molecular identification of yeast

Polymerase chain reactions (PCR) were carried out directly from yeast colonies with standardized growth of up to 5 days (young cultures), in which a portion of the biomass of the isolates was added in microtubes with 10 µL of sterile PCR water and subjected to a temperature shock of 95.0 °C for 5 minutes using the 2720 Thermal Cycler (Applied Biosystems, Foster city, CA, USA). After that, amplification of regions D1/D2 of the 26S ribosomal gene was carried out using primers NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3'), as described by Kurtzman & Robnett (1997). PCR reactions were performed in a 50 µL a volume containing 5 µL buffer (10X), 2.5 µL $MgCl_2$ (2.5 mM), 1 µL primers NL1 and NL4 (0,5 µM), 0.8 µL dNTPs (1.25 mM), 0.4 µL Taq polymerase (5U), 10 µL DNA (10 ng), and 29.3 µL PCR water to the final volume. The amplification conditions consisted of an initial denaturation of 95.0 °C for 2 min, followed by 35 denaturation cycles (95.0 °C / 30s), annealing (56.0 °C / 1 min), extension (72.0 °C / 1 min) and a final 10 min elongation step at 72.0 °C in the thermal cycler.

The PCR products were evaluated by 1% agarose gel electrophoresis and purified. Nucleotide sequences were determined using the Sanger method on the ABI platform (*Applied Biosystems Life Technologies*). In addition, phylogenetic identification was checked using Chromas v2.6.6 software, followed by comparison of the sequences obtained with sequences deposited in the NCBI GenBank database (http://www.ncbi.nml.nih.gov) with higher similarities. The sequence was aligned using the Bioedit software v7.0.0 (Hall 1999) and the phylogenetic trees were obtained by the Neighbor-Joining method MEGA X 10.1 (Kumar et al. 2018).

RESULTS

Enzyme screening

A total of 144 yeast isolates recovered from lichen samples were evaluated for hydrolase activity. Among these, a total of 109 (76%) isolates produced at least one of the hydrolase enzymes tested, confirmed by the presence of degradation halos (hydrolysis) around the screened colonies. Protease producers were the most abundant among the yeast isolates with 41% positive isolates (n=59), followed by 40% cellulase (n=58), 39% esterase (n=57), 20% lipase (n=29), 16% amylase (n=23), and 14% pectinases (n=20) producers. Moreover, a high abundance was observed of proteases, cellulases, and esterases by yeasts recovered mainly from *Lecania brialmontii*, *Usnea aurantiacoatra,* and *Polycauliona candelaria* (Table I, Figure 3).

Table I. Number of yeasts isolated from Antarctic lichens with positive enzymatic activity (n).

Lichen species (Geographic location code)	Isolates n* (**)	Amylase (n)	Cellulase (n)	Esterase (n)	Lipase (n)	Pectinase (n)	Protease (n)
Cladonia metacorallifera Asahina (L30)	6 (6)	0	3	1	2	0	6
Lecania brialmontii (Vain.) Zahlbr. (L19)	33 (23)	10	11	13	6	3	14
Mastodia tessellata (Hook. f. & Harv.) Hook. f. & Harv. (L14)	5 (4)	1	1	1	2	0	2
Polycauliona regalis (Vain.) Hue (L15)	9 (5)	2	2	4	1	2	4
Polycauliona candelaria (L.) Frödén, Arup & Søchting (L4, L6, L12, L13)	22 (14)	2	11	7	4	4	5
Rhizocarpon geographicum (L.) DC. (L23)	2 (2)	0	0	0	1	0	1
Sphaerophorus globosus (Huds.) Vain. (L10)	5 (4)	1	2	1	1	2	3
Umbilicaria decussata (Vill.) Zahlbr. (L16)	2 (2)	0	0	0	1	0	1
Usnea antarctica Du Rietz (L36)	1 (1)	0	0	0	0	0	1
Usnea aurantiacoatra (Jacq.) Bory (L1, L2, L3, L7, L25, L31, L33, L34, L35)	50 (41)	7	24	24	11	6	19
Usnea capillacea Motyka (L11, L32)	9 (7)	0	4	6	0	3	3
Total positive (n)	144 (109)	23	58	57	29	20	59
% positives	75%	16%	40%	39%	20%	14%	41%

***n: number of isolates evaluated.**
**** : number of positive isolates.**

Yeasts identification

Among the isolates evaluated, seventy-six were identified (Supplementary Material - Table SI). Most isolates were basidiomycetes with a predominance of the genera *Vishniacozyma* (n=32, 42%), *Cystobasidium* (n=10, 13%), *Bannozyma* (n=8, 10%), *Mrakia* (n=6, 8%), and *Holtermanniella* (n=3, 4%). The most frequent species were *Vishniacozyma victoriae* (n=27, 35%), *Cystobasidium alpinum* (n=3, 4%), *Mrakia niccombsii* (n=3, 4%), *Cystobasidium laryngis* (n=2, 3%), *Bannozyma yamatoana* (n=2, 3%), *Holtermanniella nyarrowii* (n=2, 3%) and *Glaciozyma martinii* (n=2, 3%). Isolates classified as ascomycetes belonged to the genus *Candida* (n=3, 4%), mainly *C. davisiana* (n=2, 3%). The remaining yeasts were grouped in *Hannaella phetchabunensis*, *Leucosporidium creatinivorum*, *Phenoliferia glacialis*, *Saitozyma flava, Dioszegia* sp., *Kondoa* sp., *Papiliotrema* sp. and *Phaeotremella* sp. The low similarity of the 26S ribosomal gene sequence of isolate 11.L16

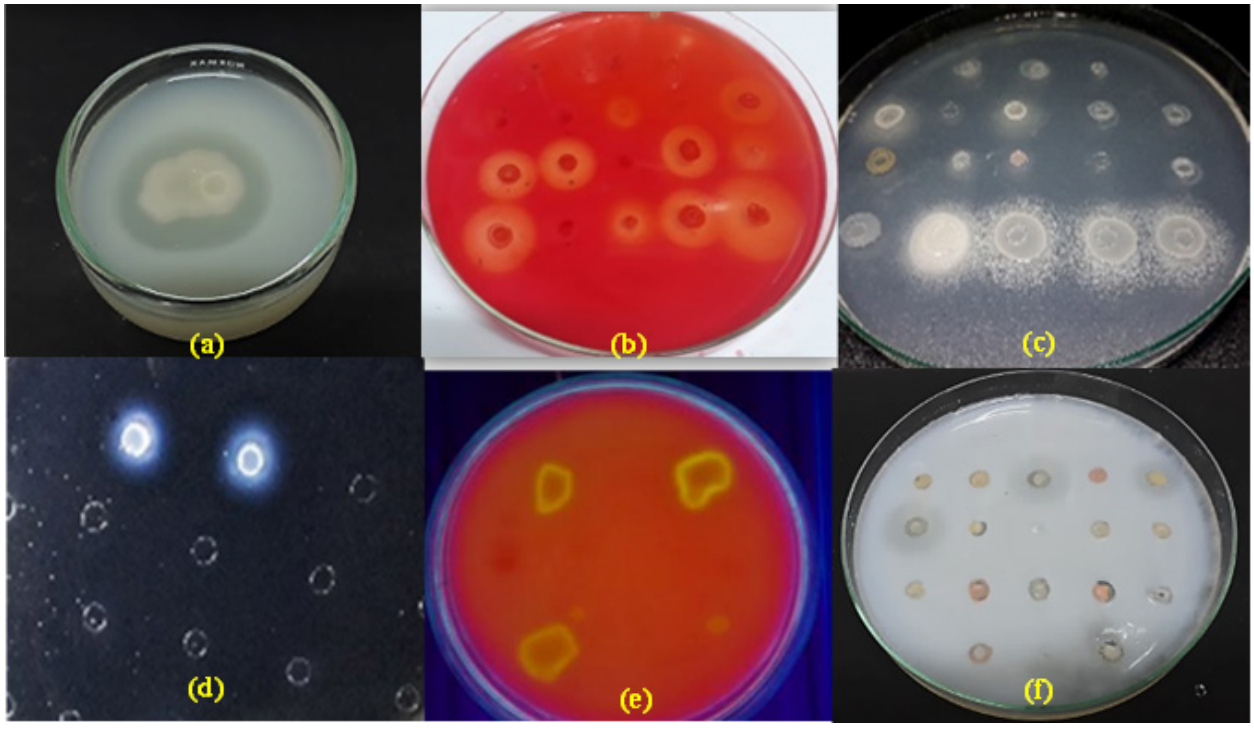

Figure 3. Positive production of extracellular enzymes by Antarctic yeasts isolated from different species of lichen: (a) protease, (b) cellulase, (c) esterase, (d) amylase, (e) lipase, (f) pectinase. The isolates were incubated at 15.0 ° C for 7 days.

with sequence of reference organisms deposited in Genbank suggest that this isolate may represent a new genus of the order Tremellales. The identified yeasts were mainly associated with lichens *U. aurantiacoatra*, *L. brialmontii*, *P. candelaria* and *P. regalis* (Supplementary Material - Figure S1).

Enzymatic activity was frequent among yeasts of the genus *Vishniacozyma*, with predominance of *V. victoriae*, whose isolates exhibited activity for all hydrolases evaluated: cellulases (n=21, 36%), esterases (n=17, 30%), proteases (n=11, 19%), pectinases (n=9, 45%), amylases (n=7, 30%), and lipases (n=4, 14%) (Table II). While the isolates belonging to the genus *Cystobasidium*, *C. alpinum* and *C. laryngis*, were able to produce five hydrolases among them, except pectinases. *Bannozyma* was the third most frequent genus, with activity for lipases, proteases and esterases. Isolates of *M. niccombsii* produced more proteases and esterases (Table SII). The ascomycete *Candida* sp. produced all hydrolases evaluated, except for lipase and pectinase.

No isolate produced all six enzymes under the conditions analyzed, as indicated in Venn diagram (Figure 4). Only four isolates produced five of them, including *S. flava* 4.L4, *V. victoriae* 2.L15 and *V. victoriae* T.L19. Fourteen isolates (12%) showed activity for four of the analyzed hydrolases, while twenty-four (22%) showed enzymatic activity for three of them, such as *H. nyarrowii* (positive cellulase, esterase and pectinase). Activity for two enzymes was found in 31% (n=34) of positive yeasts, including *H. phetchabunensis*, *Kondoa* sp., *L. creatinivorum* and *P. glacialis.*

The Enzymatic Index (IE) fluctuated from the lowest value of 1.0 to 5.3 among all positive isolates (Table SII). The yeast *V. victoriae* (strains F.L11, G.L11 and I.L11) (both IE=5.3) and *V. victoriae* 2.L4 (IE=4.1) showed the best cellulase

Table II. Number of species of yeast isolated from Antarctic lichens with positive enzymatic activity (n): amylase (Amy), cellulase (Cell), esterase (Est), lipase (Lip), pectinase (Pec), and protease (Prot).

Yeast (n)	Amy.	Cell.	Est.	Lip.	Pec.	Prot.
Ascomycota						
Candida davisiana (2)	-	2	1	-	-	2
Basidiomycota						
Bannozyma arctica (1)	-	-	1	1	-	-
Bannozyma sp. (5)	1	1	2	2	1	3
Bannozyma yamatoana (2)	-	-	-	-	1	1
Cystobasidium alpinum (3)	1	1	-	1	-	1
Cystobasidium laryngis (2)	-	-	2	1	-	1
Cystobasidium sp. (5)	1	3	3	3	-	1
Dioszegia sp. (1)	-	-	1	-	-	1
Glaciozyma martinii (2)	1	2	1	-	-	2
Hannaella phetchabunensis (1)	-	-	1	-	1	-
Holtermanniella nyarrowii (2)	-	2	1	-	1	-
Holtermanniella sp. (1)	-	-	-	-	-	1
Kondoa sp. (1)	-	1	-	1	-	-
Leucosporidium creatinivorum (1)	-	-	1	-	-	1
Leucosporidium sp. (1)	-	-	1	-	-	1
Mrakia gelida (1)	-	-	-	-	-	1
Mrakia niccombsii (3)	1	1	2	-	-	3
Mrakia sp. (2)	-	1	-	-	1	-
Papiliotrema sp. (2)	-	-	-	1	2	2
Phaeotremella sp. (2)	-	-	-	1	-	-
Phenoliferia glacialis (1)	-	-	1	-	-	1
Phenoliferia sp. (1)	-	-	-	1	-	-
Saitozyma flava (1)	1	1	-	1	1	1
Tremellales (1)	-	-	-	-	-	1
Vishniacozyma sp. (5)	1	3	4	-	-	3
Vishniacozyma victoriae (26)	7	21	17	4	9	11

activities. The latter isolate also exhibited the high performance for extracellular protease production with an IE of 2.4, followed by *C. laryngis* 15.L15 (IE=2.2). For esterase, the IEs ranged from 1.1 to the highest value of 3.6 for *Vishniacozyma* sp. AA.L19 demonstrating the most expressive activity for this enzyme. In the screening of lipases, the mean was IE=1.3 among positive isolates, with the most significant value being 3.0 for the unclassified isolate 19.L15. For amylase activity, the most significant IEs ranged from 2.8 to 1.6, respectively for *Bannozyma* sp. 4.L10 and *C. alpinum* G.L19. Thus, *H. phetchabunensis* 1.L2 (IE=2.4) and *V. victoriae* 2.L3 (IE=3.8) exhibited the halos highest pectinase activities.

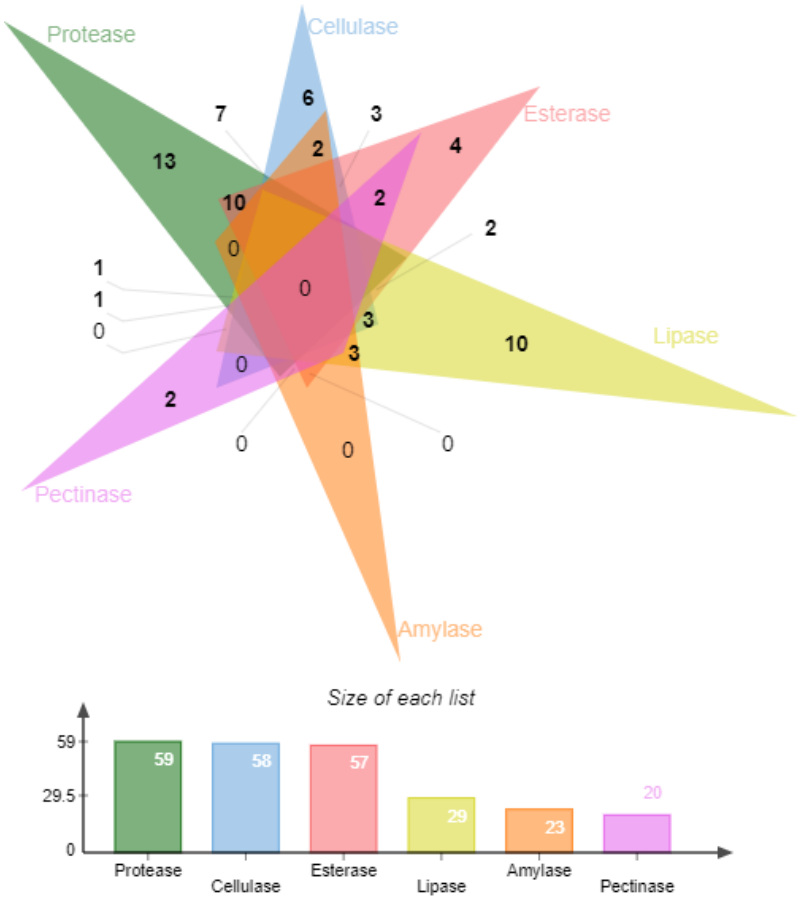

Figure 4. Venn diagram showing the distribution of enzymatic activity (protease, cellulase, esterase, lipase, amylase, and pectinase) among the yeasts isolated from Antarctic lichens.

DISCUSSION

Most Antarctic fungi that showed exoenzyme activity have been isolated from soils, macroscopic algae, water and marine sediments (Vaz et al. 2011, Martorell et al. 2019), little is known about lichens as a source of exoenzyme producing yeasts. This is one of the few studies that did screened lichens-associated yeasts in the search for hydrolases, together with the study by Duarte et al. (2013) who analyzed the production of lipases and proteases by basidiomycetic yeasts from terrestrial samples, including lichens.

In this work, all six evaluated hydrolases were detected in 76% of the yeasts tested, which showed prominent activities for extracellular proteases, cellulases and esterases. Proteases were produced by 41% of the isolates tested here and by 60% of the isolates obtained from *Lecania brialmontii* L19. The action of protease can help yeasts to capture amino acids, providing part of the maintenance of the plasma membrane and other cellular molecules necessary for metabolism, as well as contributing to the flow of carbon in the Antarctic ecosystem (Vero et al. 2019). Protease activity by Antarctic yeasts has already been reported in several studies such as *Leucosporidium antarcticum* 171 (Turkiewicz et al. 2003) and *Rhodotorula mucilaginosa* L7 (Lario et al. 2015). In our study, yeasts were identified included *Vishniacozyma victoriae*, *Bannozyma yamatoana* and *Mrakia niccombsii*.

About 39% esterase positive isolates were found among the yeast recovered from Antarctic lichens. The search for esterase is relatively common in Antarctic yeasts, and producing strains include *Cystobasidium laryngis*, *Exophialla xenobiotica*, *Holtermanniella* sp., *Leucosporidium scottii*, *Mrakia* sp., *Pichia*

caribbica and *Vishniacozyma* sp. (Vaz et al. 2011, Carrasco et al. 2012, Martorell et al. 2017). In this work, representatives of *V. victoriae*, *M. niccombsii*, *B. yamatoana* and *H. phetchabunensis* were added among the esterase-producing yeast, mainly due to the scarce reports on the last three species. Esterases are differentiated due to their catalytic action on ester bonds of lipids with short chain fatty acids, which appears to be important for capturing lipid compounds (Vero et al. 2019).

On the other hand, lipases hydrolyze bonds of lipids with long chain fatty acids. Both enzymes enable access to phospholipids, glycerols and fatty acids necessary for the maintenance of the membranes under the cold conditions of Antarctica (Duarte et al. 2018). About 29 isolates (20%) tested positive for the extracellular lipases visualized by the fluorescent halos under UV light. Most active yeasts were basidiomycetes (especially *Cystobasidium* sp., *Bannozyma* sp. and *Kondoa* sp.) and an ascomycete, *Candida* sp. Duarte et al. (2013) obtained similar data, with 84.5% of the positive yeasts belonging to Basidiomycota. Lipases are relatively abundant enzymes in Antarctic yeasts, such as *Mrakia blollopis* (Tsuji et al. 2013), and *L. scottii* (Duarte et al. 2015, 2021).

In this report, positive isolates for the screening of amylases and pectinases were around 16% and 14%, respectively. These molecules have already been screened in previous studies from soil yeasts and marine samples (Vaz et al. 2011, Carrasco et al. 2016). The evaluation of amylolytic and pectinolytic activity at 15.0 °C performed in this work and the favorable results, are in accordance with the data by Martinez et al. (2016). Lipases, esterases and proteases are often produced by Antarctic yeasts, mainly due to the crucial role they play in maintaining the membrane of these microorganisms. However, the remaining hydrolases found catalyze the breakdown of less common macromolecules found in Antarctica, which correspond to cellulose, starch and pectin from plant cells. Cellulase and pectinase represent decisive roles in carbon cycling by catalyzing the breakdown of bonds of plant molecules (Vero et al. 2019), mainly cellulases.

The works conducted by Vaz et al. (2011) and Carrasco et al. (2016) also detected cellulases in Antarctic yeasts. Similar percentage of cellulase-positive isolates was found in yeasts from soils close to lichens and *Deschampsia antarctica*, with bigger halos for *Gueomyces pullulans*, *V. victoriae* and *M. frigida* (Martorell et al. (2017). However, none of the yeasts reported by Martinez et al. (2016) isolated from water samples and Antarctic soils showed cellulase production. In our study, cellulases were confirmed in 40% of isolated yeasts; these enzymes are active on glycosidic bonds of cellulose, the main component of plant cells. Although Antarctica environment is limited to some bryophytes and two native angiosperms, lichens often form extensive and complex communities on the surface of these plants, hence the activity of these associated fungi may be involved in capturing additional glucose source through decomposition processes of these plants (Vero et al. 2019). The high activity for cellulases among the tested isolates highlights the importance they may have for this purpose.

About 15% (n=24) of the isolates showed activity for four of the six exoenzymes analyzed here. Exoenzymes production by yeasts offers a strategy for survival in Antarctica, in addition to the colonization of the lichen microecosystem where they find stability. In previous works, only *Usnea* (*U. antarctica* and *U. auranticoatra*) and *Ramalina tenebrata* lichens were investigated for the presence of associated yeasts (Santiago et al. 2015, Duarte et al. 2016). Here, the species *U. auranticoatra* was the main one for the isolation of hydrolase-producing yeasts (n=41), this relative abundance is expected, as *Usnea* corresponds to one of the most abundant genera throughout Antarctica. However, *L.*

brialmontii L19 was the one that represented both the largest number of isolates and enzyme activity (n=23), in addition to the species *P. candelaria*, *U. capillacea*, *C. metacorallifera* and *P. regalis*. This is one of the first reports about yeasts associated with thallus of these species of Antarctic lichens.

Spribille et al. (2016) have reported basidiomycetic yeasts from Cyphobasidiales in the *Bryoria* (*B. fremontii* and *B. tortuosa*) lichen cortex. Cystobasidiomycete yeasts have also been found in association with lichen *Cladonia* (Černajová & Škaloud 2019). While Tuovinen et al. (2019) demonstrated the occurrence of the *Tremella* genome in lichens from *Letharia*, in Montana - USA. These are reports that have strengthened the hypothesis that yeasts are ubiquitous in macroliquens, which are an important microhabitat for fungal dispersion on a global scale. However, investigations of the presence and roles that yeasts play in lichens are still scarce (Duarte et al 2016, Muggia et al. 2017). Thus, taking into account that a large part of Antarctic ecosystems is occupied by lichens, together with the advantageous colonization of basidiomycetic yeasts in this environment, the ability of these microorganisms to metabolize different sources of molecules by secreting exoenzymes can be one of the contributions to such symbioses.

In this sense, Antarctic lichens proved to be suitable substrates for yeasts, which mostly belonged to the phylum Basidiomycota, with notoriety in the production of extracellular hydrolases. With regard to the diversity of the positive yeasts identified, the data obtained reinforce previous results that have shown the singular prevalence of basidiomycetes species with high biotechnological potential to be explored in the cold Antarctic region, as they present greater metabolic plasticity, which help them to propagate in this environment. This plasticity is due to the increase of unsaturated fatty acids in its plasma membranes that make it more flexible against the effects of low temperatures. This plasticity enables them to survive in cold, acidic and oligotrophic habitats with nutrient scarcity, which seems to be limiting for ascomycetes yeasts (Deming & Young 2017, Yurkov 2018).

The yeasts *V. victoriae*, *C. laryngis* and *Mrakia* sp. were recurrent among the isolates obtained, which is in accordance with data from Duarte et al. (2016) in Antarctic lichens. Particularly *Vishniacozyma* is a cosmopolitan genus common in cold areas, with representatives found in all aquatic and terrestrial substrates, with *V. victoriae* being one of the most abundant species (Garcia et al. 2012, Buzzini et al. 2017). This species also prevailed in the studies conducted by Santiago et al. (2015) and Duarte et al. (2013). Together with this species, the yeasts of *Mrakia* and *Cystobasidium* form a large part of the Antarctic mycobiota, well adapted to their conditions. The strains found significantly demonstrated high potential for enzymatic activity (Carvalho et al. 2019).

Some new basidiomycete yeasts found in the lichens studied here, such as *B. yamatoana*, *Hannaella phetchabunensis*, *Kondoa* sp., *Holtermanniella nyarrowi*, *M. niccombsii* and *Saitozyma flava,* also represent the first hydrolytic enzymes-producing yeasts in this group. In general, the extracellular enzymes produced by basidiomycetic yeasts due to their particular structural and molecular properties are important in microbial propagation in extreme environments, but at the same time, they also have many promising chemical applications in the sectors of production of food, tissues and clinical uses. Therefore, the search for microorganisms in unusual habitats has increased, since they express atypical compounds with specific particular properties for a variety of biotechnology interests (Garcia et al. 2012, Bruno et al. 2019).

In view of the results, yeasts showed production of extracellular enzymes active, confirming their potential as a source of hydrolases, especially proteases, cellulases and esterases. A significant amount of yeasts from Antarctic lichens was obtained, revealing them as suitable substrates for the presence of these microorganisms. Taxonomic evaluation indicated that the yeast isolates obtained belong to different species, mainly of the phylum Basidiomycota and including *V. victoriae*, *C. alpinum, C. laryngis*, *B. yamatoana* and *M. niccombsii*. Thus, these data confirm lichens as a source of yeasts hydrolase-producing, reinforcing the potential of these microorganisms to be studied in the search of new catalytic molecules in polar regions that may be useful in promising biotechnological applications.

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SUPPLEMENTARY MATERIAL

Figure S1.
Tables SI, SII.

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