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RESEARCH PAPER

Bioprospection of cold-adapted yeasts with biotechnological potential from Antarctica

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The aim of this study was to investigate the ability to produce extracellular hydrolytic enzymes at low temperature of yeasts isolated from 25 de Mayo island, Antarctica, and to identify those exhibiting one or more of the evaluated enzymatic activities. A total of 105 yeast isolates were obtained from different samples and 66 were identified. They belonged to 12 basidiomycetous and four ascomycetous genera. Most of the isolates were ascribed to the genera *Cryptococcus*, *Mrakia*, *Cystobasidium*, *Rhodotorula*, *Gueomyces*, *Phenoliferia*, *Leucosporidium*, and *Pichia*. Results from enzymes production at low temperatures revealed that the Antarctic environment contains metabolically diverse cultivable yeasts, which represent potential tools for biotechnological applications. While most the isolates proved to produce 2-4 of the investigated exoenzymes, two of them evidenced the six evaluated enzymatic activities: *Pichia caribbica* and *Guehomyces pullulans*, which were characterized as psycrotolerant and psycrophilic, respectively. In addition, *P. caribbica* could assimilate several n-alkanes and diesel fuel. The enzyme production profile and hydrocarbons assimilation capacity, combined with its high level of biomass production and the extended exponential growth phase make *P. caribbica* a promising tool for cold environments biotechnological purposes in the field of cold-enzymes production and oil spills bioremediation as well.

KEYWORDS
antarctica, biotechnology, enzymes, yeasts

1 | INTRODUCTION

Antarctica constitutes one of the most extreme environments on Earth, presenting several extreme climatic conditions including low temperature, low humidity, and high solar irradiation [1]. Considering both, continental and maritime regions, <2% of the Antarctic continental surface corresponds to ice-free land areas. Antarctic soils are mainly oligroptrophics [2], except for areas where some macroorganisms, as penguins or elephant seals settled down and breed. Precipitations are scarce and winds are strong and extremely variable. All these factors determine a wide range of extreme soil microhabitats which differs in temperature, moisture, organic carbon contents and levels of N, P, and other macronutrients [3].

Such plethora of different environmental conditions constitute a huge challenge for survival of terrestrial microorganisms, that need to possess several physiological and metabolic adaptation to cope with these extreme conditions. In the recent decades, Antarctic extremophilic microorganisms have been the focus of numerous

investigations, that centered the attention on psychrophilic bacteria, archaea, algae [4], and more rarely on fungi [5–8].

These cold-adapted microorganisms can grow at 0 °C and are classified as psychrophilic when their optimum and maximum temperatures for growth are ≤15 °C, or as psychrotolerant (psychrotrophic) if their optimum temperature for growth is above 15 °C [9]. As such, microorganisms have adapted their vital cellular processes to thrive in cold environments [10], they make essential contributions to nutrient cycling and organic matter mineralization in polar regions via a special class of extracellular enzymes known as cold-adapted or cold-active enzymes [11]. Because these enzymes have a higher catalytic efficiency than their mesophilic counterparts at temperatures below 20 °C and display unusual substrate specificities [11], they are attractive candidates for industrial processes requiring high enzymatic activity at low temperatures. Cold-adapted enzymes include amylase, cellulases, invertase, inulinase, proteases, lipases, and isomerases among others, which are used in the food, biofuel, and detergent industries [12]. There are several examples of cold enzymes currently used in diverse biotechnological and industrial processes; and some examples have been listed by Sarmiento et al [13].

The aerobic biodegradation of many components of petroleum hydrocarbons, including n-alkanes, aromatic and polycyclic aromatic hydrocarbons (PAHs), at low temperatures has been reported in Arctic, Alpine, and Antarctic environments [14,15]. A wide variety of bacteria, fungi, and algae can metabolize aliphatic and aromatic hydrocarbons [16]. Filamentous fungi are known for their potential to degrade PAHs [17]. There is, however, little information about the hydrocarbon-degradative potential of yeasts, despite this, microeukariotic organisms could be useful and efficient tools for developing biotechnological processes of bioremediation of fuels spilled on soils of extremely cold regions.

Because of their great potential in biotechnological applications, from which the previously mentioned represents only a small sample, cold-adapted microorganisms have become increasingly studied in recent years. Despite the amazing diversity of polar and subpolar microorganisms that is being revealed in the last years, the real potential of them is scarcely known. In addition, among the most isolated and studied microorganisms from cold environments, yeasts only constitute a minor fraction [18], nevertheless yeasts recently isolated from Antarctica were evaluated for their lipids accumulation with excellent results [19] and some cold adapted yeasts from Patagonia were evaluated for their plant growth enhancing features [20].

In the north-west of the Antarctic Peninsula, 25 de Mayo/King George is the largest island within the South Shetland archipelago. The local climate is typical of the peri-Antarctic islands: wet, windy, and cold, with an average temperature of 1-3 °C in the warmest month, −7 °C in the coldest month and with very few sunny days indeed [21].

Studies on these islands indicate that minimal soil temperature in winter is commonly buffered by overlying snow and remain above about −5 to 9 °C, even though short-term minimal air temperatures may be much lower. Similarly, short-term maximal soil temperatures in the range 14-26 °C are frequently measured [22].

Based on this background, the objective of this study was the isolation and characterization of psychrophilic/psychotropic yeast from an Antarctic site located at WAP and characterized by their high biological diversity. We specially focused our attention on amylase, cellulase, pectinases, proteases, and estearase activity of a collection of 105 isolates. After that, two yeasts showing all the tested activities were selected and their kinetic properties were analyzed. Additionally, their ability to degrade the dominant n-alkanes present in fuels-contaminated Antarctic sois (nC11, nC12, nC13, nC14, nC16) and gasoil was tested.

2 | MATERIALS AND METHODS

2.1 | Sampling

Soil near lichens and *Deschampsia Antarctica* and mud samples were collected during the 2013/2014 austral summer (December 2013-March 2014) nearby Argentinean Scientific Research Station, Carlini Base, located on the Potter Cove, 25 de Mayo Island (62°14′18″S, 58°40′00″W) (Fig. 1A).

Samples were collected from a range of areas around the Cove, including an ornithogenic soil in a penguin rockery (Stranger Point); on the beach, around a refuge house for scientist (Elephant refuge) and a largely pristine and naturally vegetated area, Tres Hermanos hill. Also, soils from two human-impacted areas in Carlini station facilities (under the main dining room and near the gas oil tanks) and a largely pristine and naturally vegetated area (Tres Hermanos hill) were sampled (Fig. 1B).

Samples (around 10 g) were taken from soil at a depth of 0-10 cm, using a sterile spatula. After collection, the samples were stored in sealed sterile bags or sterile flasks and immediately returned to the research station, where they were refrigerated at 4 °C, and subsequently treated for incubation and isolation.

2.2 | Yeasts isolation

For yeast isolation purposes, a portion of each soil sample (approximately 0.5 g) was excised under sterile conditions, using a sterile spoon or spatula directly spread onto Petri dishes containing agarized Yeast Morfology Medium (YM) diluted 1:10, composition in g/L: yeast extract 0.3, malt extract 0.3, peptone 0.3, dextrose 0.5, and agar 20.0. To promote the growth of yeasts instead of bacteria pH was adjusted to 4.5. The plates were incubated at 15 °C for 18-25 days under natural lighting conditions. Actively growing

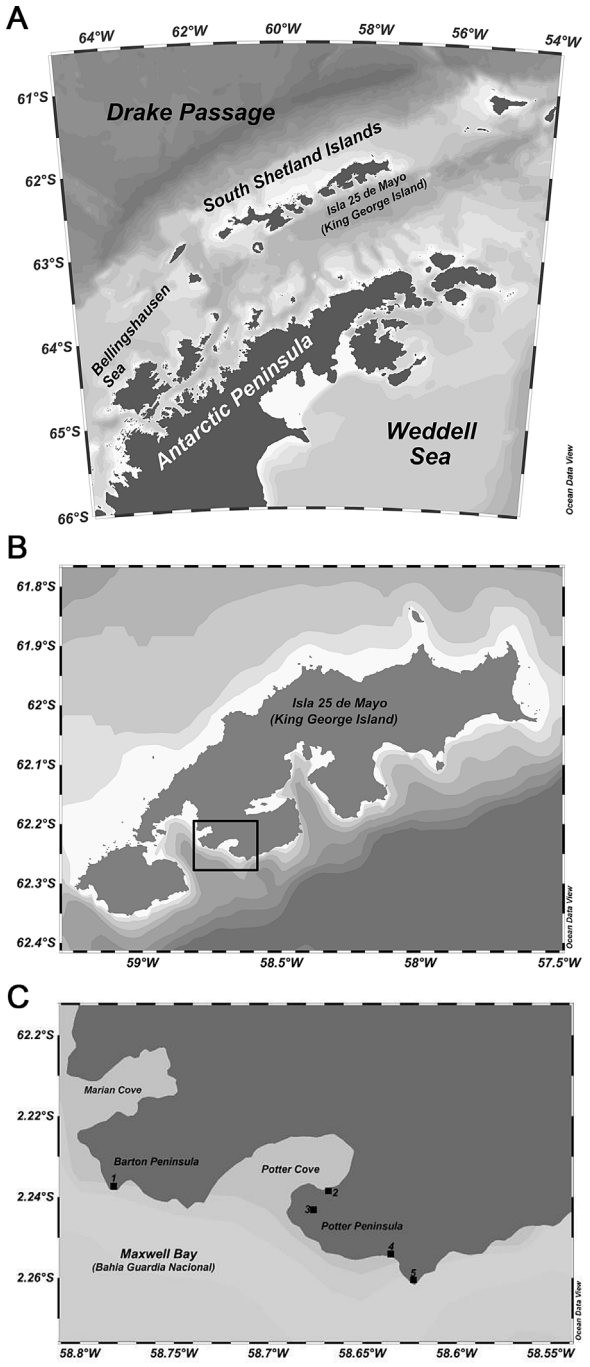

FIGURE 1 The studied area in King George Island/Isla 25 de Mayo, South Shetland Islands, with indication of the sampling site, Potter Peninsula (62°14′18″S, 58°40′00″W) (A and B). Sampling sites (C): 1, Nesting penguins in Barton Peninsula; 2, Carlini Station facilities; 3, Tres Hermanos Hill; 4, Elefant Refuge; 5, Stranger Point

colonies were then taken from the plates and subcultured onto fresh YM 1:10 agar plates as individual isolates. Yeast isolates were stored in the Microbiological Resources Center Culture Collection (MIRCEN) of PROIMI-CONICET Institute, San Miguel de Tucumán, Argentina and in the Culture Collection in the Argentinean Antarctic Institute (IAA). All yeast strains were maintained on isolation medium agar plates (without antibiotics) at 4 °C and transferred monthly.

2.3 | Screening of lytic enzymes production

Seven lytic activities were tested on the isolates growing on solid media at 15 °C: amylase, cellulose, lipase, esterase, protease, pectinase, and xylanase. In all cases, exoenzymatic activity was quantified as the diameter of the halo in mm (of either coloration or decoloration) around the colony.

2.3.1 | Amylolytic activity

The cells were grown in YM medium (1:10) with starch (2 g L^{-1}) instead of glucose. After incubation the plates were flooded with 1 ml of iodine solution, and positive activity was defined as a clear halo around the colony on a purple background (halos were measured in millimeters) [23].

2.3.2 | Cellulase activity

The cells were grown in YM medium (1:10) with carboxymethylcellulose (5 g L^{-1}) instead of glucose [8]. The plates were flooded with Congo red solution (1 mg ml^{-1}), which was poured off after 15 min. The plates were then flooded with 1 M NaCl for 15 min. Positive cellulase activity was defined as a clear halo around the colony on a red background.

2.3.3 | Protease activity

The cells were grown in YM medium (1:10) supplemented with skim milk (10 g L^{-1}). Positive protease activity was defined as a clear halo (produced by the casein degradation) around the colony on a white opaque background [24].

2.3.4 | Pectinase activity

The cells were grown in YM medium (1:10), pH 7.0, containing 10 g L^{-1} pectin. The plates were flooded with 10 g L^{-1} CTAB (Cetyl trimethylammonium bromide), and positive activity was indicated by a clear halo around the colony on a white background [25].

2.3.5 | Esterase activity

The cells were grown in medium composed of (in g L^{-1}): bacto peptone, 10; NaCl, 5; $CaCl_2 \cdot 2H_2O$, 4 and Tween 80, 10. Esterase activity was evidenced as a white precipitate around the colony [8].

2.3.6 | Lipase activity

The cells were grown in YM medium (1:10) supplemented with olive oil (4% v/v) and rhodamine B 0.01%. Lipase activity was indicated as fluorescent halos around the colonies under UV exposure [26].

2.3.7 | Xylanase activity

The cells were grown in YM medium (1:10) with xylane (5 g L^{-1}) instead of glucose. After incubation the plates were flooded with 1 ml of iodine solution, and positive activity was defined as a clear halo around the colony on a purple background (halos were measured in millimeters) [27].

2.4 | Molecular identification of selected isolates

Only the isolates that presented one or more of the evaluated enzymatic activities ($n = 66$) were selected for identification. Genomic DNA extraction was performed according to Libkind et al [28]. The divergent domain at the 5′ end of the LSU rDNA gene (around 600 bp) was symmetrically amplified with primers NL-1 (5′-GCATATCAATAAGCGGAGGAAAAG) and NL-4 (5′-GGTCCGTGTTTCAAGACGG) according to standard methods, as described by Kurtzman [29]. Sequences were analyzed and edited, when necessary, using DNA Dragon software [30]. All isolates were sequenced and their DNA sequences were submitted to GenBank under Accession Numbers listed in Table 1. Strains identification was performed by comparison with the GenBank (only type strains). A ≥99% identity criterion was employed to identify strains at the species level. Taxonomy was checked against Kurtzman [29]. Sequences showing 97-98% identity were tentatively identified to the genus level. Sequences showing less than 97% identity were considered unidentified.

2.5 | Growth studies

2.5.1 | Growth temperature range

The effect of temperature on the growth of the selected strains was investigated on agar plates. Loopful of microbial cells (pre-grown on YM agar plates) were used to inoculate two replicates per strain and temperature on YM agar. The plates were incubated at 5, 10, 15, 20, 25, 30, and 37 °C. Growth was monitored up to an incubation time of 21 days.

2.5.2 | Growth of selected yeasts at low temperature in liquid culture

Growth kinetics of the selected strains were evaluated in 250 ml-Erlenmeyer flasks containing 50 ml of YNB (Yeast Nitrogen Base) medium supplemented with glucose (20 g L^{-1}) and $(NH4)_2SO_4$ (2.5 g L^{-1}). About 5 mL of yeast suspensions ($OD_{550} = 0.8$), prepared from a 48 h-old YM broth culture were used to inoculated the flasks. Incubations were carried out in a rotatory shaker at 15 °C and 250 rpm for 120 h. Abiotic controls were performed in flasks with the same medium without inoculum. Cultures were aseptically sampled at different times and growth estimated spectrophotometrically at 550 nm. Culture samples were centrifuged for 10 min at 6500×*g* and pH and residual glucose were measured from supernatants (comercial kit GT Lab).

2.5.3 | Growth of selected yeasts on hydrocarbons supplemented media

Selected isolates were inoculated in liquid YNB (Yeast Nitrogen Base) medium supplemented with 1% (w/v) of diesel fuel, or different alkanes (undecane [nC11], dodecane [nC12], tridecane [nC13], and tetradecane [nC14]) as C and energy source. Cultures were incubated at 15 °C and 250 rpm. Growth was evaluated spectrophotometrically at 550 nm and compared with growth in the control media (without any C source). As hydrocarbons in liquid medium prevent the possibility to measure growth directly from the culture broth, 1 ml aliquot was centrifuged for 10 min at 13,000 rpm, supernatant was discharged and the pellet was resuspended in 1 ml of distilled water to measure DO at 550 nm.

2.6 | Statistical analysis

All values and data points presented in this work represents the means of at least triplicate determinations of independent assays. Data were analyzed using the GraphPad InStat Instant Biostatistics package version 3.0. Statistical analysis was conducted using Minitab (Minitab Inc., State College, PA, USA). Biomass yields differences were tested by Kruskal-Wallis test, a non-parametric one-way ANOVA test. A critical value of 0.1 was chosen arbitrarily based on the size and type of experiment [31].

3 | RESULTS

3.1 | Identification of culturable antarctic yeasts

A total of 105 isolates were obtained after isolation and culture procedures. As only yeasts presenting one or more of the evaluated enzymatic activities ($n = 66$) were considered for further studies, 66 out of 105 isolates were selected for identification (Table 1). Based on those selected isolates, soil and lichen samples yielded the higher number of strains (25 and 24, respectively), followed by mud ($n = 14$) and *Deschampsia* ($n = 3$).

Two different databases were used for identification of isolates, NCBI and AFTOL. As was explained above, sequences showing 97-98% identity were tentatively identified to the genus level whereas those showing less than 97% identity were considered unidentified and a new blast with non-type material was performed to approximate to the genera or, at least, confirm if it was an asco- or basidiomycetous yeast (Table 2).

From the 66 selected isolates we identified seventeen different species (Table 2) as well as twelve basidiomycetous and four ascomycetous genera. Most of the isolates from the basiodiomycetous group belongs to genera *Cryptococcus* ($n = 20$), *Mrakia* ($n = 11$), *Cystobasidium* ($n = 6$), *Rhodotorula* ($n = 4$) and *Gueomyces* ($n = 3$); *Phenoliferia* ($n = 3$) and *Leucosporidium* ($n = 3$). In the case of the ascomycetous group, most isolates belonged to *Pichia* ($n = 8$).

3.2 | Enzymatic evaluation of all isolates

Considering that in this study yeasts were selected based on their hydrolytic enzyme activity, and as it was previously

TABLE 1 Enzime activity of selected yeast

		Enzyme activities (mm)[a]						
Isolate number	**Presuntive identification**	**Amilase**	**Cellulase**	**Lipase**	**Esterase**	**Protease**	**Pectinase**	**Xylanase**
88	*Cryptococcus adeliensis*	2	-	3	3	1	7	-
27	*Cryptococcus gastricus*	1	-	-	-	-	-	1
84	*Cryptococcus gastricus*	1	-	-	-	3	-	-
176	*Cryptococcus gilvescens*	-	-	-	3	-	-	-
2	*Cryptococcus victoriae*	1	5	-	3	-	-	-
6	*Cryptococcus victoriae*	1	2	-	6	-	-	-
9	*Cryptococcus victoriae*	1	2	-	5	-	-	-
56	*Cryptococcus victoriae*	1	4	-	6	-	-	-
80	*Cryptococcus victoriae*	1	4	-	3	-	-	-
103	*Cryptococcus victoriae*	1	6	-	2	-	-	1
105	*Cryptococcus victoriae*	1	3	-	-	-	-	-
107	*Cryptococcus victoriae*	1	4	-	4	-	-	-
131	*Cryptococcus victoriae*	1	5	-	4	-	-	-
155	*Cryptococcus victoriae*	2	5	-	4	-	-	-
163	*Cryptococcus victoriae*	1	3	-	5	-	-	-
185	*Cryptococcus victoriae*	-	-	-	3	-	-	-
251	*Cryptococcus victoriae*	1	3	-	5	-	-	-
278	*Cryptococcus victoriae*	1	3	-	5	-	-	-
291	*Cryptococcus victoriae*	1	3	-	5	-	-	-
322	*Cryptococcus victoriae*	1	5	-	4	-	-	-
60	*Cystobasidium laryngis*	-	-	-	5	-	-	-
130	*Cystobasidium laryngis*	-	-	-	4	-	-	-
217	*Cystobasidium laryngis*	-	-	-	5	-	-	-
309	*Cystobasidium laryngis*	-	-	-	3	-	-	-
318	*Cystobasidium laryngis*	-	-	-	2	-	-	-
341	*Cystobasidium laryngis*	-	-	-	4	-	-	-
90	*Dioszegia hungarica*	1	1	-	2	-	-	-
54	*Fellomyces penicillatus*	4	-	-	-	-	-	3
286	*Glaciozyma litoralis*	-	-	-	-	3	-	-
28	*Guehomyces pullulans*	3	5	3	5	3	4	4
37	*Guehomyces pullulans*	3	3	3	6	3	3	6
53	*Guehomyces pullulans*	4	4	3	6	3	3	6
134	*Holtermanniella* sp.	2	-	-	6	-	-	-
273	*Leucosporidium creatinivorum*	-	-	2	3	4	-	-
275	*Leucosporidium creatinivorum*	-	-	1	4	5	-	-
276	*Leucosporidium creatinivorum*	1	-	1	1	4	-	-
1	*Mrakia frigida*	1	2	2	-	3	6	-
92	*Mrakia frigida*	-	5	2	-	4	7	-
190	*Mrakia frigida*	-	4	2	-	2	5	-
259	*Mrakia frigida*	1	4	2	4	4	7	-
260	*Mrakia frigida*	1	3	1	-	3	6	-
261	*Mrakia frigida*	-	1	2	-	1	5	-
263	*Mrakia frigida*	1	4	2	-	3	3	-
264	*Mrakia frigida*	-	3	2	3	5	5	-
7	*Mrakia frígida*	1	2	2	-	4	6	-
153	*Mrakia frígida*	1	2	2	3	2	7	-

(Continues)

TABLE 1 (Continued)

Isolate number	Presuntive identification	Enzyme activities (mm)[a]						
		Amilase	Cellulase	Lipase	Esterase	Protease	Pectinase	Xylanase
162	*Mrakia frígida*	1	3	1	2	-	1	-
307	*Nadsonia commutata*	-	-	-	-	1	-	-
159	*Phenoliferia glacialis*	-	-	1	-	-	-	2
166	*Phenoliferia glacialis*	-	-	1	3	-	-	-
201	*Phenoliferia glacialis*	-	1	-	-	-	-	3
8	*Pichia caribbica*	2	4	3	5	3	2	4
59	*Pichia caribbica*	-	3	1	2	4	-	-
161	*Pichia caribbica*	2	4	3	5	3	2	4
168	*Pichia caribbica*	1	3	-	2	-	-	1
171	*Pichia caribbica*	2	4	3	6	3	2	4
205	*Pichia caribbica*	2	4	2	4	1	2	3
256	*Pichia caribbica*	2	2	1	1	-	1	2
288	*Pichia caribbica*	-	-	2	2	4	-	-
128	*Protomyces inouyei*	-	-	1	1	-	-	-
248	*Rhodotorula mucilaginosa*	-	-	-	-	1	-	-
172	*Rhodotorula muscorum*	-	-	1	-	7	-	-
174	*Rhodotorula muscorum*	-	-	2	3	6	-	-
97	*Rhodotorula* sp.	-	-	1	-	6	-	-
31	Unidentified yeast	4	5	4	5	4	8	4
211	Unidentified yeast	-	-	1	1	-	1	-

[a]Exoenzymatic activity was quantified as the diameter (in mm) of the halo (of either coloration or decoloration) around the colony as explained in the materials and methods section

emphasized that oligotrophic microorganisms are usually related to the ability to degrade a broad spectrum of substrates, while copiotrophic microorganisms are related to the efficient degradation of easily accessible substrates [7], both the isolation scheme and the screening process lead us toward selecting the most promising yeasts for future cold-environment biotechnological process.

In this work, isolates showing multiple extracellular enzymatic activities were obtained from sampling sites with the higher content of organic matter, either as a consequence of the human impact (soil that suffered fuels spills from the storage tanks throughout the years) or from natural origin (complex substrates as those present in soils around lichens and *Deschampsia* or mud near creeks).

Amylase activity was produced by 58% ($n = 59$) of the isolates. *Guehomyces pullulans* and *Fellomyces penicillatus* strains showed the largest haloes. Cellulase activity was evidenced by 58% ($n = 59$) of the isolates, too. The larger and comparable haloes were exhibited by *G. pullulans*, *C. victoriae*, *P. caribbica*, and *M. frigida*, all yeasts previously isolated from Antarctica. In this case, yeasts with the biggest cellulolytic haloes were isolated from soil around lichen, a cellulose and lignin-rich substrate. Protease activity was presented in 46% of the isolates, and the the biggest haloes were exhibited by *Leucosporidiella* sp., *Leucosporidium creatinivorum, Rhodotorula* sp., and *M. frigida.* Pectinase was produced by 31% of the isolates with the biggest haloes presented by *P. caribbica*, *M. frigida*, and *G. pullulans. P. caribbica*, and *G. pullulans* also presented the wider haloes for xylanase activity. Nevertheless, this was the enzymatic activity less produced by the isolates, with only 20% of positive results, as also reported by Carrasco et al [8], nevertheless this activity was previously described in *Cryptococcus* species isolated from Antarctica [32] it seems that it is not frequently evaluated in cold-adapted micro-organisms as most of reports are in mesophilic or thermophilic conditions [33]. Lipase activity was detected in 49% of the isolates (with *P. caribbica, M. frigida, Leucosporidiella* sp., and *G. pullulans*, presenting the biggest haloes), but esterase, another lipolytic enzyme, was produced by 72%, with the biggest haloes presented by *P. caribbica* and *G. pullulans*, *C. laryngis* and *Cryptococcus victoriae*.

3.3 | Effect of temperature on growth of yeasts

The seven strains previously selected (*P. caribbica* [isolates 8, 161, 171, and 205] and *G. pullulans* [isolates 28, 37, and 53]) were grown in complex medium (YM) at temperatures ranging from 5 to 37 °C. Only one strict psychrophile (isolate 37, *G. pullulans*), showing no growth above 25 °C [34], was

TABLE 2 Molecular identification of selected yeast isolates

Isolate number	Accesion number	NCBI identification	Identity	Query cover	Total score	Number of bp analized	Accesion number	Presuntive identification
1	KY033101	*Mrakia frigida* CBS 5270 28S rRNA, partial sequence; from TYPE material	99	95	1077	586	*NG_042346.1*	*Mrakia frigida*
2	KY033102	*Cryptococcus victoriae* 26S ribosomal RNA gene, partial sequence	99	95	1070	586	*AF363647.1*	*Cryptococcus victoriae*
6	KX773555	*Cryptococcus victoriae* 26S ribosomal RNA gene, partial sequence	99	96	1059	586	*AF363647.1*	*Cryptococcus victoriae*
7	KY033103	*Mrakia frigida* CBS 5270 28S rRNA, partial sequence; from TYPE material	99	96	1079	591	*NG_042346.1*	*Mrakia frígida*
8	KY033104	*Pichia caribbica* strain NRRL Y-27274 26S ribosomal RNA gene, partial sequence	99	94	979	548	*EU348786.1*	*Pichia caribbica*
9	KX773561	*Cryptococcus victoriae* 26S ribosomal RNA gene, partial sequence	99	96	941	528	AF137600.1	*Cryptococcus victoriae*
27	KY033105	*Cryptococcus gastricus* 26S ribosomal RNA gene, partial sequence	99	96	979	537	AF137600.1	*Cryptococcus gastricus*
28	KY033106	*Guehomyces pullulans* isolate AFTOL-ID 1958 25S large subunit ribosomal RNA gene, partial sequence	99	97	1081	596	*EF551318.1*	*Guehomyces pullulans*
31	KY052792	*Pichia guilliermondii* strain p 26S ribosomal RNA gene, partial sequence	87	73	571	522	*EU188617.1*	Unidentified yeast
37	KX773531	*Guehomyces pullulans* CBS 2532 28S rRNA, partial sequence; from TYPE material	99	99	1081	595	*EF551318.1*	*Guehomyces pullulans*
53	KX773537	*Guehomyces pullulans* CBS 2532 28S rRNA, partial sequence; from TYPE material	99	99	985	558	*EF551318.1*	*Guehomyces pullulans*
54	KX773543	*Fellomyces penicillatus* 26S ribosomal RNA gene, partial sequence	99	97	1044	590	*AF177405.1*	*Fellomyces penicillatus*
56	KY033107	*Cryptococcus victoriae* 26S ribosomal RNA gene, partial sequence	99	96	1068	582	*AF363647.1*	*Cryptococcus victoriae*
59	KX773549	*Pichia caribbica* strain NRRL Y-27274 26S ribosomal RNA gene, partial sequence	99	99	987	541	*NG042640.1*	*Pichia caribbica*
60	KY033108	*Cystobasidium laryngis* strain CBS 2221 26S ribosomal RNA gene, partial sequence	99	96	1055	588	*AF189937.1*	*Cystobasidium laryngis*
80	KY033109	*Cryptococcus victoriae* 26S ribosomal RNA gene, partial sequence	99	96	1061	581	*AF363647.1*	*Cryptococcus victoriae*
84	KX773556	*Cryptococcus gastricus* 26S ribosomal RNA gene, partial sequence	99	98	963	530	*AF137600.1*	*Cryptococcus gastricus*
88	KY033110	*Cryptococcus adeliensis* CBS 8351 28S rRNA, partial sequence; from TYPE material	99	96	1064	583	NG042353.1	*Cryptococcus adeliensis*
90	KY033111	*Dioszegia hungarica* CBS 4214 28S rRNA, partial sequence; from TYPE material	99	96	1055	586	NG042350.1	*Dioszegia hungarica*
92	KY033112	*Mrakia frigida* CBS 5270 28S rRNA, partial sequence; from TYPE material	99	97	1085	594	*NG_042346.1*	*Mrakia frigida*
97	KY033113	*Rhodotorula muscorum* 26S ribosomal RNA gene, partial sequence	98	97	1014	577	*AF070433.1*	*Rhodotorula* sp.
103	KY033114	*Cryptococcus victoriae* 26S ribosomal RNA gene, partial sequence	99	98	1068	582	*AF363647.1*	*Cryptococcus victoriae*
105	KY033115	*Cryptococcus victoriae* 26S ribosomal RNA gene, partial sequence	99	97	1072	590	*AF363647.1*	*Cryptococcus victoriae*
107	KY033116	*Cryptococcus victoriae* 26S ribosomal RNA gene, partial sequence	99	96	1055	581	*AF363647.1*	*Cryptococcus victoriae*
128	KY033117	*Protomyces inouyei* 28S rRNA, partial sequence; from TYPE material	99	96	1003	562	NG042406.1	*Protomyces inouyei*
130	KY033118	*Cystobasidium laryngis* strain CBS 2221 26S	99	96	1037	583	*AF189937.1*	*Cystobasidium*

(Continues)

TABLE 2 (Continued)

Isolate number	Accesion number	NCBI identification	Identity	Query cover	Total score	Number of bp analized	Accesion number	Presuntive identification
		ribosomal RNA gene, partial sequence						*laryngis*
131	KY033119	*Cryptococcus victoriae* 26S ribosomal RNA gene, partial sequence	99	96	1068	585	*AF363647.1*	*Cryptococcus victoriae*
134	KX773548	*Holtermanniella takashimae* partial 26S rRNA gene, strain HB 982	97	99	1005	591	*AY138478.1*	*Holtermanniella* sp.
153	KY033120	*Mrakia frigida* CBS 5270 28S rRNA, partial sequence; from TYPE material	99	97	1068	591	*NG_042346.1*	*Mrakia frígida*
155	KY033121	*Cryptococcus victoriae* 26S ribosomal RNA gene, partial sequence	99	96	1061	581	*AF363647.1*	*Cryptococcus victoriae*
159	KY033122	*Phenoliferia glacialis* strain A19 26S ribosomal RNA gene, partial sequence	99	97	1042	577	*EF151258.1*	*Phenoliferia glacialis*
161	KY033123	*Pichia caribbica strain* NRRL Y-27274 26S ribosomal RNA gene, partial sequence	99	94	996	546	*EU348786.1*	*Pichia caribbica*
162	KY033124	*Mrakia frigida* CBS 5270 28S rRNA, partial sequence; from TYPE material	99	96	1079	591	*NG_042346.1*	*Mrakia frígida*
163	KY033125	*Cryptococcus victoriae* 26S ribosomal RNA gene, partial sequence	99	96	1057	582	*AF363647.1*	*Cryptococcus victoriae*
166	KX773532	*Phenoliferia glacialis* strain A19 26S ribosomal RNA gene, partial sequence	99	97	1055	581	*EF151258.1*	*Phenoliferia glacialis*
168	KY033126	*Pichia caribbica strain* NRRL Y-27274 26S ribosomal RNA gene, partial sequence	99	94	992	544	*EU348786.1*	*Pichia caribbica*
171	KX773538	*Pichia caribbica strain* NRRL Y-27274 26S ribosomal RNA gene, partial sequence	99	95	965	535	*EU348786.1*	*Pichia caribbica*
172	KX773544	*Rhodotorula muscorum* 26S ribosomal RNA gene, partial sequence	99	98	1044	576	*AF070433.1*	*Rhodotorula muscorum*
174	KX773550	*Rhodotorula muscorum* 26S ribosomal RNA gene, partial sequence	99	98	1029	560	*AF070433.1*	*Rhodotorula muscorum*
176	KY033127	*Cryptococcus gilvescens* 26S ribosomal RNA gene, partial sequence	99	96	976	532	*AF181547.1*	*Cryptococcus gilvescens*
185	KY033128	*Cryptococcus victoriae* 26S ribosomal RNA gene, partial sequence	99	97	1024	580	*AF363647.1*	*Cryptococcus victoriae*
190	KX773557	*Mrakia frigida* CBS 5270 28S rRNA, partial sequence; from TYPE material	99	96	1086	591	*NG_042346.1*	*Mrakia frigida*
201	KY033129	*Phenoliferia glacialis* strain A19 26S ribosomal RNA gene, partial sequence	99	97	1055	581	*EF151258.1*	*Phenoliferia glacialis*
205	KY033130	*Pichia caribbica* strain NRRL Y-27274 26S ribosomal RNA gene, partial sequence	99	95	1003	550	*EU348786.1*	*Pichia caribbica*
211	KX773565	*Phenoliferia glacialis* strain A19 26S ribosomal RNA gene, partial sequence	96	46	725	459	*EF151258.1*	Unidentified yeast
217	KX773533	*Cystobasidium laryngis* strain CBS 2221 26S ribosomal RNA gene, partial sequence	99	98	1044	588	*AF189937.1*	*Cystobasidium laryngis*
248	KX773558	*Rhodotorula mucilaginosa* 26S ribosomal RNA gene, partial sequence	100	97	1031	558	*AF070432.1*	*Rhodotorula mucilaginosa*
251	KX773560	*Cryptococcus victoriae* 26S ribosomal RNA gene, partial sequence	99	97	1055	588	*AF363647.1*	*Cryptococcus victoriae*
256	KY033131	*Pichia caribbica* strain NRRL Y-27274 26S ribosomal RNA gene, partial sequence	99	94	996	546	*EU348786.1*	*Pichia caribbica*
259	KY033132	*Mrakia frigida* CBS 5270 28S rRNA, partial sequence; from TYPE material	99	96	1085	594	*NG_042346.1*	*Mrakia frigida*
260	KY033133	*Mrakia frigida* CBS 5270 28S rRNA, partial sequence; from TYPE material	99	96	1092	594	*NG_042346.1*	*Mrakia frigida*
261	KY033134	*Mrakia frigida* CBS 5270 28S rRNA, partial sequence; from TYPE material	99	97	1086	594	*NG_042346.1*	*Mrakia frigida*

(Continues)

TABLE 2 (Continued)

Isolate number	Accesion number	NCBI identification	Identity	Query cover	Total score	Number of bp analized	Accesion number	Presuntive identification
263	KY033135	*Mrakia frigida* CBS 5270 28S rRNA, partial sequence; from TYPE material	99	96	1085	594	*NG_042346.1*	*Mrakia frigida*
264	KX773540	*Mrakia frigida* CBS 5270 28S rRNA, partial sequence; from TYPE material	99	98	1061	586	*NG_042346.1*	*Mrakia frigida*
273	KX773552	*Leucosporidium creatinivorum* CBS 8620 28S rRNA, partial sequence; from TYPE material	98	96	1044	572	*NG_042375.1*	*Leucosporidium creatinivorum*
275	KY033136	*Leucosporidium creatinivorum* CBS 8620 28S rRNA, partial sequence; from TYPE material	99	97	1057	582	*NG_042375.1*	*Leucosporidium creatinivorum*
276	KX773559	*Leucosporidium creatinivorum* CBS 8620 28S rRNA, partial sequence; from TYPE material	98	97	1070	579	*NG_042375.1*	*Leucosporidium creatinivorum*
278	KY033137	*Cryptococcus victoriae* 26S ribosomal RNA gene, partial sequence	99	97	1055	584	AF363647.1	*Cryptococcus victoriae*
286	KY033138	*Glaciozyma litoralis* genomic DNA containing ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene, isolate K94b	99	92	1016	554	HF934009.1	*Glaciozyma litoralis*
288	KY033139	*Pichia caribbica* strain NRRL Y-27274 26S ribosomal RNA gene, partial sequence	99	95	1009	550	*EU348786.1*	*Pichia caribbica*
291	KY033140	*Cryptococcus victoriae* 26S ribosomal RNA gene, partial sequence	99	95	1059	579	*AF363647.1*	*Cryptococcus victoriae*
307	KY033141	*Nadsonia commutata* strain NRRL Y-7950 26S ribosomal RNA gene, partial sequence	99	94	998	547	*KC254858.1*	*Nadsonia commutata*
309	KY033142	*Cystobasidium laryngis* strain CBS 2221 26S ribosomal RNA gene, partial sequence	99	97	1053	587	*AF189937.1*	*Cystobasidium laryngis*
318	KY033143	*Cystobasidium laryngis* strain CBS 2221 26S ribosomal RNA gene, partial sequence	98	96	1053	587	*AF189937.1*	*Cystobasidium laryngis*
322	KY033144	*Cryptococcus victoriae* 26S ribosomal RNA gene, partial sequence	100	96	1079	584	*AF363647.1*	*Cryptococcus victoriae*
341	KY033145	*Cystobasidium laryngis* strain CBS 2221 26S ribosomal RNA gene, partial sequence	99	96	1051	579	*AF189937.1*	*Cystobasidium laryngis*

present in the group. The other selected strains were considered as psychrotolerants.

3.4 | Kinetic growth in cold conditions

To learn more about the two yeasts species having members with all the studied catabolic activities (*G. pullulans* 37 and *P. caribbica* 171) their kinetic growth was evaluated using easily assimilating carbon and nitrogen sources, glucose and ammonium sulfate, respectively. As was mentioned above, *G. pullulans* 37 and *P. caribbica* 171 were characterized as strict psychrophilic and psycrotolerant respectively, for that reason they were selected to compare their behavior at 15 °C and in liquid culture. Growth kinetic, pH, and glucose concentration in liquid cultures of *G. pullulans* 37 and *P. caribbica* 171 are shown in Fig. 2. Both strains started the exponential phase at 8 h of culture, but surprisingly, *G. pullulans* 37 reached stationary phase at 41 h while *P. caribbica* 171 continued growing until 121 h. At the end of the culture, biomass (expressed as dry weight), reached 10.15 and 3.21 g L^{-1} for *P. caribbica* and *G. pullulans*, respectively. In relation to the maximum specific growth rate (μ_m), *G. pullulans* 37 presented a slightly higher value than *P. caribbica* 171 (0.0576 and 0.0554, respectively), nevertheless there was not significant differences between the maximum specific growth rate of both strains. In the two cultures, an acidification, characteristic of yeasts cultures, was observed from the start. Nevertheless *P. caribbica* 171 reached pH values as low as 1.86, which might indicate a high production of some organic acids during culture. Regarding glucose consumption, this was complete in both cultures, at 72 and 96 h for *G. pullulans* 37 and *P. caribbica*, 171 respectively suggesting a higher efficient for substrate to biomass conversion (Yx/s) for the *P. caribbica* 171.

3.5 | Growth on hydrocarbons as C source

Only *P. caribbica* 171 was able to growth using all the hydrocarbons evaluated, undecane (nC11), dodecane (nC12), tridecane (nC13), tetradecane (nC14), and diesel fuel at

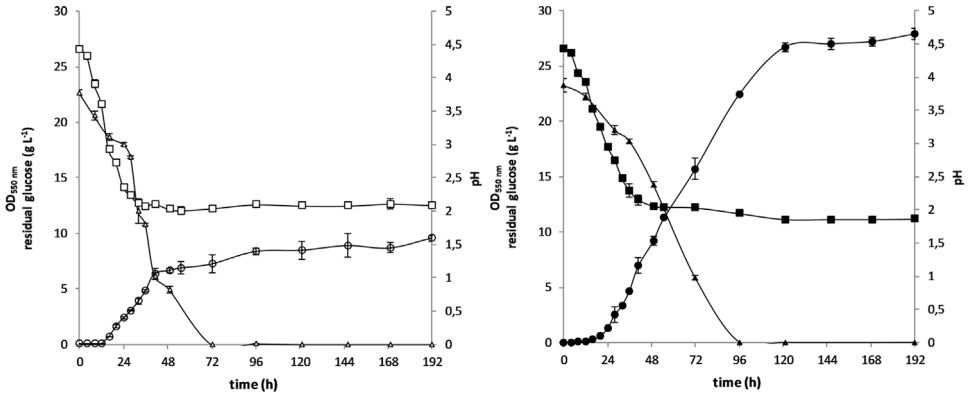

FIGURE 2 Growth, pH and residual glucose kinetics of *P. carabbica*171 (right) and *G. pullulans*37 (left) in YNB medium complemented with glucoseat 15 °C and 250 rpm. Growth: *P. carabbica* (●) and *G. pullulans* (○); pH: *P. carabbica* (■) and *G. pullulans* (□); and residual glucose: *P. caribbica* (▲) and *G. pullulans* (Δ)

15 °C in liquid culture. At 7 days of culture DO600 nm was 1.7, 0.97, 0.80, 0.73, and 0.98 for nC11, nC12, nC13, nC14, and diesel fuel, respectively, evidencing a negative relationship between the carbon chain length and yeast growth.

4 | DISCUSSION

The predominance of basidiomycetous in cold environments, such as the Antarctica, had been linked to their ability to produce polysaccharide capsules and the increase in the proportion of unsaturated fatty acids compared to the saturated fatty acids in the plasmatic membrane [3,35]. Nevertheless, the higher proportion of basidiomycetous species detected in this work might be also explained based on the low carbon content of the isolation culture media employed. This feature could have biased results towards oligotrophic, slow-growing, metabolically diverse yeasts, characteristics mainly found among basidiomycetous genera.

Based on literature, yeasts dwelling in Antarctic and sub-Antarctic maritime and terrestrial habitats belong mainly to the genera *Cryptococcus, Candida, Rhodotorula,* and *Mrakia* [7,8,36]. In this work other genera, as *Cistobasidium, Fellomyces, Guehomyces, Leucosporidium, Meyerozyma, Nadsonia, Phenoliferia, Dioszegia, Protomyces, Glaciozyma,* and *Pichia* were also isolated.

From the 21 *Cryptococcus* strains, 13 were identified as *Cryptococcus victoriae*. This species was first described for the Antarctica by Montes et al [37], who isolated it from a soil sample of Southern Victoria Land. The presence of this species in cold areas of the world became more apparent with successive isolations from extremely cold water-related environments, such as glacial ice from the Arctic [38] and the Italian Alps [39,40]. They were also obtained from a range of different habitats in temperate regions [41]. The presence of *C. victoriae* in aquatic environments outside the polar areas was less frequently documented. In summary, *C. victoriae* is a species that inhabits very diverse environments and climatic zones, and can adapt to a variety of environmental conditions. Also, members of genus *Cryptococcus* were isolated repeatedly from soil samples and some researchers have described them as the most important life form in Antarctic desert soils [42]. From permanent cold environments, many other genera (approximately 53) were described, but in smaller proportions [8,41,43–46].

The cold adapted yeasts *Mrakia* spp. were found in several permanent cold environments as the Arctic, Alaska, the Italian Alps, Patagonia, and Antarctica [47]. In this work, eleven isolates from all kind of samples (soil, mud, lichen, and *Deschampsia*) were identified as *M. frigida*. The genus *Mrakia* accounts for about 24% of the culturable yeast from Antarctic soil [48]. Moreover, it was previously reported that about 35% of culturable fungi isolated from lake sediment and soil of East Antarctica were *Mrakia* spp. [49]. These reports suggested that *Mrakia* spp. are the dominant culturable fungi in East Antarctica and the most adaptive to the Antarctic area. In the case of *M. frigida*, it was also isolated from deadwood or insect [50], soil [51], and frozen food [52].

Rhodotorula and *Cystobasidium,* the other genera frequently found in Antarctica as well as in several others cold regions [34], comprise anamorphic basidiomycetous yeasts isolated from different habitats, including aquatic, hypersaline, and psychrophilic environments [53]. In this work, 10 isolates belong to these genera, and were also isolated from different substrates and locations.

Other taxa found in different samples near Carlini Base and previously isolated in Antarctica and reported as cold adapted are *Pichia caribbica* (7), *Guehomyces pullulans* (4), *Leucosporidium creatinivorum* (3), *Phenoliferia glacialis* (4), *Nadsonia commutata* (1), *Fellomyces penicillatus* (1), *Dioszegia hungarica* (1), and *Holtermanniella* sp. (1) [54,55].

Based on the available literature, this is the first report of *G. litoralis* and *P. inouyei* in Antarctica. *G. litoralis*, is a newly discovered species, which was isolated in the Arctic zone, specifically in the White Sea [56]. *P. inouyei* was also

previously isolated in the Arctic zone, but never in Antarctica [57]. These results represent an important contribution to the knowledge of the Antarctic mycodiversity.

In this work, special attention was paid to lipases and esterases producers, as these are activities related to hydrocarbons degradation [12], a relevant catabolic ability with potential application in bioremediation processes of polluted cold environments. When the sites from which the lipase/sterase positive isolates were obtained were analyzed, it can be observed that several producer yeasts (*C. Adeliensis*, *G. pullulans*, *P. caribbica*, and *Ph. glacialis*) were isolated from soil near the diesel fuel storage tanks. Although microorganisms able to efficiently degrade oil hydrocarbons have been isolated from uncontaminated environments [14], their numbers, including fungi, significantly increase in oil-contaminated soils. In this sense, Aislabie et al [58] attributed the significant enhancement in numbers of culturable yeasts and filamentous fungi in oil-contaminated cold soils to the important role of these microorganisms in the degradation of hydrocarbons or their metabolites.

When the whole enzymatic production of the isolates was analyzed, seven yeasts were selected as they presented all the evaluated activities. The selected strains were isolates 8, 28, 37, 53, 161, 171, and 205, which were classified as *G. pullulans* (basidiomycetous) or *P. caribbica* (ascomycetous). Five out of the seven isolates were obtained from the same sample, a soil near fuels storage. As was mentioned above; this data should not be surprising, as this soil had a higher amount of hydrocarbons, accumulated as a consequence of the leaks of gasoil from the tanks throughout the years. The chronic presence of hydrocarbons selected a microbiota with dominance of microorganisms able to tolerate and catabolize these recalcitrant organic compounds, as was previously reported for the studied area [59]. These yeasts were selected for further studies based on their biotechnological potential, primarily for hydrocarbons bioremediation in cold environment.

The predominance of psychrotolerant fungi in surface soils from cold environments has been previously noted and, as mentioned, it is attributable to seasonal and diurnal increases in soil temperature due to solar radiation [9] that favors the dominance of the "eurythermal" cold adapted microorganism over the stenothermal ones represented by the strict psychrophiles.

Several yeast species were reported in the literature as being able to use hydrocarbons as carbon sources. These hydrocarbon-degrading yeasts belong mainly to the genera *Candida, Clavispora, Debaryomyces, Leucosporidium, Lodderomyces, Metschnikowia, Pichia, Rhodosporidium, Rhodotorula, Sporidiobolus, Sporobolomyces, Stephanoascus, Trichosporon,* and *Yarrowia* [60]. In the case of *P. caribbica* it was previously reported to produce a biosurfactant [61] which could be related with its ability to assimilate n-alkanes and gasoil as carbons sources.

In summary, one-hundred and five isolates obtained from Antarctica were tested for lytic enzyme synthesis in solid media. The cold-adapted yeast producing one or more of the evaluated enzymes were characterized as oligotrophic, slow-growing and metabolically diverse basidiomycetes. It is also the first report of *G. litoralis* and *P. inouyei* in Antarctica. The selection of yeasts with at least one cold-active enzymatic activity enhances their potential for biotechnological/industrial exploitation. While most the isolates exhibited 2-4 enzyme activities, two of them exhibited the six enzyme activities: *P. caribbica* 171 and *G. pullulans* 37. The study of the selected strains in liquid medium proved that *P. caribbica* 171 is a psycrotolerant yeasts whereas *G. pullulans* 37 resulted a psycrophilic microorganism.and, respectively. Also, *P. caribbica* 171 can assimilate a several number of n-alkanes and diesel fuel. The enzyme production profile and hydrocarbons assimilation combined with its high level of biomass production, extended exponential phase and the possible production of organic acids make *P. caribbica* 171 a promising tool for cold environments biotechnological purposes in the field of cold-enzymes production or in the oil spills bioremediation as well.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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