Microbial Ecology (2019) 77:12–24
https://doi.org/10.1007/s00248-018-1217-x

FUNGAL MICROBIOLOGY

Fungi from Admiralty Bay (King George Island, Antarctica) Soils and Marine Sediments

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Received: 3 December 2017 / Accepted: 31 May 2018 / Published online: 19 June 2018

Abstract

Extreme environments such as the Antarctic can lead to the discovery of new microbial taxa, as well as to new microbial-derived natural products. Considering that little is known yet about the diversity and the genetic resources present in these habitats, the main objective of this study was to evaluate the fungal communities from extreme environments collected at Aldmiralty Bay (Antarctica). A total of 891 and 226 isolates was obtained from soil and marine sediment samples, respectively. The most abundant isolates from soil samples were representatives of the genera *Leucosporidium*, *Pseudogymnoascus*, and a non-identified Ascomycota NIA6. *Metschnikowia* sp. was the most abundant taxon from marine samples, followed by isolates from the genera *Penicillium* and *Pseudogymnoascus.* Many of the genera were exclusive in marine sediment or terrestrial samples. However, representatives of eight genera were found in both types of samples. Data from non-metric multidimensional scaling showed that each sampling site is unique in their physical-chemical composition and fungal community. Biotechnological potential in relation to enzymatic production at low/moderate temperatures was also investigated. Ligninolytic enzymes were produced by few isolates from root-associated soil. Among the fungi isolated from marine sediments, 16 yeasts and nine fungi showed lipase activity and three yeasts and six filamentous fungi protease activity. The present study permitted increasing our knowledge on the diversity of fungi that inhabit the Antarctic, finding genera that have never been reported in this environment before and discovering putative new species of fungi.

Keywords Extremophiles · Fungal diversity · Marine mycology · Maritime Antarctica · Cold-adapted enzymes

Introduction

The Antarctic environment is characterized by low temperatures, high UV incidence, dryness, freeze and thaw cycles, low nutrient content, and high salinity, in addition to long periods of darkness [1–3]. Despite these extreme conditions, many microorganisms can thrive in this environment. Fungi and bacteria play great ecological roles in the Antarctic environment, but their diversity information is still poorly explored [4]. Fungi that inhabit marine sediments and soil work as decomposers and are dominant components of the biomass in Antarctic ecosystems [5, 6].

Antarctic microorganisms, considered extremophiles, developed adaptation mechanisms, including high concentrations of unsaturated membrane lipids, antifreeze proteins, and enzymes that are active at low temperatures known as cold-adapted enzymes [7–10]. Cold-adapted enzymes are active at low and moderate temperatures, require lower activation energy, and are stable at higher temperatures (the inactivation temperature usually is higher than the catalysis optimal temperature, unlike the mesophilic analogs). All these advantages are attractive for industrial processes because they can decrease energy costs. Besides this, in processes conducted

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00248-018-1217-x) contains supplementary material, which is available to authorized users.

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with enzymes that are extremely efficient at low temperatures, the contamination by other microorganisms can be avoided [11, 12].

Oxidative enzymes can be applied in a wide range of industrial processes in the food and textile industries. Besides, they have pharmaceutical and nanotechnological applications and can also be used for effluent treatment [13–15]. Hydrolytic enzymes such as proteases account for approximately 60% of the world enzyme trade and are used for treating industrial rejects and in the production of pharmaceuticals [16, 17].

Considering the ecological relevance, the potential of fungi to produce oxidases and hydrolases and the properties of cold-adapted enzymes, fungi from the Antarctic environment could be considered as interesting microbial resources to be exploited and biotechnologically applied [18]. In this sense, the aim of the present study was to characterize and compare the fungal diversity in marine sediment and in different Antarctic soil samples and also to evaluate the capability of these fungi to produce oxidative (terrestrial fungi) and hydrolytic (marine fungi) enzymes.

Methods

Sampling Site

Soil, plants, and marine sediment samples were collected in triplicate at eight different points in the Admiralty Bay region (King George Island, South Shetlands Archipelago, Maritime Antarctica) (Fig. 1) in January 2015. At Yellow Point, two different types of soil (yellow and dark brown soils) were collected (5 cm depth). At Punta Hennequin and Punta Plaza, the vascular plants *Deschampsia antarctica* and *Colobanthus quitensis* were collected with root-aggregated soil. Marine sediment samples were collected with a Van Veen grab. All samples were stored in sterile plastic bags and kept under refrigeration for up to two months until isolation took place. The triplicates were merged to form composite samples, yielding the final samples listed in Table 1.

Soil and Marine Sediment Physical and Chemical Characterization

Soil and sediment granulometry was measured by the method adapted from Camargo et al. [19]. To determine iron (Fe) and aluminum (Al) content, three different measurements were done: the dithionite-citrate-bicarbonate procedure as performed by Mehra and Jackson [20], the ammonium oxalate procedure according to McKeague and Day [21], and sodium pyrophosphate after McKeague et al. [22].

Isolation of Soil Microorganisms

Soil samples (12.5 g) and root-associated soil (6.25 g) were added to Erlenmeyer flasks containing 112.5 mL of Savitha's minimal medium [23]. As inducers for the production of ligninolytic enzymes, 0.2% of sterilized sugarcane bagasse and rice straw were added to the samples, separately. The Erlenmeyer flasks were agitated at 140 rpm and incubated at 5 °C and 15 °C for 7 days. After the incubation period, 200 μl of each sample were taken and serially diluted in a 0.085% NaCl solution. The dilutions of 10^{-1} and 10^{-3} of each sample were plated on Petri dishes. Four different culture media were used (in g L^{-1}): MA2%: 20 malt extract, 15 agar; MA2% 10× diluted; BSA: 15 malt extract, 2 yeast extract, 15 agar, 2 lactic acid (added after autoclaving), and B&K: 10 glucose, 2 peptone, 1 yeast extract, 20 agar, 4 mM guaiacol (added after autoclaving).

Isolation of Marine Sediment Microorganisms

Isolation was carried out in two steps, with and without sample enrichment. For the enrichment step, 25 g of each sample were added to Erlenmeyer flasks containing 225 mL of artificial seawater (ASW) and enzymatic inductors for lipase or protease. Olive oil 1.5% (*v*/*v*) and Tween 80 1.5% (*v*/*v*) were used as lipase inductors and Skim Milk 2% (m/v) as protease inductor. Each sample was agitated at 150 rpm and incubated at 5 °C and 15 °C for 10 days. After this period, 200 μL of the dilutions 10^{-1} and 10^{-3} were inoculated in Petri dishes on four different culture media (in g L^{-1}): PDA: 200 potato, 20 glucose, 15 agar; PDA 10× diluted; BSA, and MA - Marine Agar: 5 peptone, 1 yeast extract, 0.1 ferric citrate, 19.45 NaCl, 8.8 $MgCl_2$, 3.24 Na_2SO_4, 1.8 $CaCl_2$, 0.55 KCl, 0.16 $NaHCO_3$, 0.08 KBr, 34.0 mg L^{-1} $SrCl_2$, 22.0 mg L^{-1} HBr, 4.0 mg L^{-1} Na_2SiO_3, 1.6 mg L^{-1} NH_4NO_3, 8.0 mg L^{-1} Na_2HPO_4, 15 agar. Plates were incubated at 5 and 15 °C. Isolation without the enrichment followed the same principle: 25 g of each sample in 225 mL of artificial seawater (ASW), but without any inductor. Each sample was homogenized for 60 min at 150 rpm, at 5 and 15 °C. Then, 200 μL of the dilutions 10^{-1} and 10^{-3} were inoculated in Petri dishes in the same culture media listed above.

For both isolation processes, streptomycin (0.01 g L^{-1}) and chloramphenicol (0.1 g L^{-1}) were added to all culture media after their autoclavation. Plates were incubated up to 2 months at 5 and 15 °C to retrieve both psychrophilic and psychrotrophic fungi. Individual colonies of fungi were purified in their isolation media. Long- and medium-term preservation were carried out at − 80 and 4 °C using cryotubes with sterile 10% glycerol and water, respectively. All of the fungal isolates obtained in this study were deposited in the Microbial Resource Center Culture Collection of the São Paulo State University, Rio Claro, Brazil, under the codes CRM and LAMAI included in the sequences deposited in Genbank (see the topic *Accession numbers*).

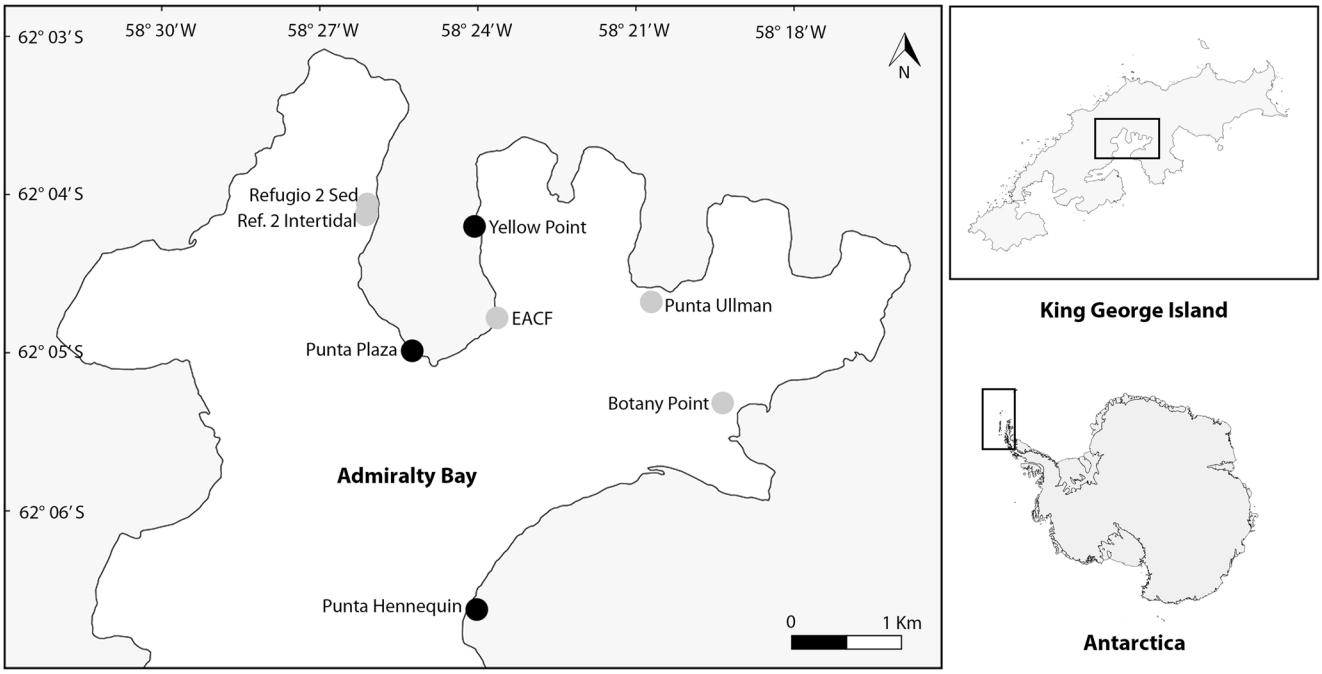

Fig. 1 Sampling sites in Aldmiralty Bay (King George Island, South Shetlands Archipelago, Maritime Antarctica)

Fungal Identification

Soil filamentous fungi were grouped in morphotypes. Fungal macromorphology was used to separate the morphotypes, and one representative of each morphotype was randomly chosen for DNA sequencing. For marine sediment fungi, all isolates had their DNA sequenced. DNA extraction of the filamentous fungi followed the method of Lacerda et al. [24]. The ITS region was amplified and sequenced with the primer pair ITS4 and ITS5 [25].

A mini satellite-primed PCR (MSP-PCR) was done to separate the soil yeasts in different fingerprints. Yeast DNA

Table 1 Data of the terrestrial and marine samples collected at Admiralty Bay

Site	Sample	Geographic coordenate	Average *T* (°C)	Average depth (m)
Yellow Point (S1)	Yellowish soil	62° 04.479′ S; 58° 23.726′ W	3.6	Superficial (5 cm)
Yellow Point (S2)	Dark brown soil	62° 04.479′ S; 58° 23.726′ W	2.5	Superficial (5 cm)
Punta Hennequin (S3)	*C. quitensis* root-associated soil	62° 07.216′ S; 58° 23.677′ W	3.6	Superficial (5 cm)
Punta Hennequin (S4)	*D. antarctica* root-associated soil	62° 07.216′ S; 58° 23.677′ W	3.5	Superficial (5 cm)
Punta Plaza (S5)	*D. antarctica* root-associated soil	62° 05.363′ S; 58° 24.691′ W	6.4	Superficial (5 cm)
Punta Plaza (S6)	*C. quitensis* root-associated soil	62° 05.363′ S; 58° 24.691′ W	6.3	Superficial (5 cm)
Botany Point (Sed1)	Marine sediment	62° 05.734′ S 58° 19.919′ W	0.6	24.7
EACF[a] (Sed2)	Marine sediment	62° 05.130′ S 58° 23.356′ W	0.4	22.9
Punta Ullman (Sed3)	Marine sediment	62° 05.015′ S 58° 20.987′ W	0.3	20.3
Refugio 2 (Sed4)	Marine sediment	62° 04.373′ S 58° 25.335′ W	0.1	20.0
Refugio 2 intertidal zone (Sed5)	Marine sediment	62° 04.341′ S 58° 25.233′ W	2.4	Superficial (5 cm)

[a] EACF in front of the Brazillian Station

extraction followed the method adapted from Sampaio et al. [26] and De Almeida [27]. MSP-PCR was performed using $(GTG)_5$ primer according to Duarte et al. [28]. One representative of each different fingerprint was randomly selected for DNA sequencing. The LSU (D1/D2) region was amplified and sequenced with the primer pair NL1 and NL4 [29]. PCR for LSU was performed according to Duarte et al. [28].

Amplicons from filamentous fungi and yeasts were purified using the enzymes Exonuclease I and Alkaline phosphatase (Thermo Scientific, Massachusetts, USA) according to the manufacturer's protocol. Samples were quantified in NanoDrop® (Thermo Scientific) and sequenced using the BigDye Terminator® v.3.1 kit (Applied Biosystems, California, USA) after the manufacturer's instructions in an ABI 3500 sequencer (Applied Biosystems). The sequencing conditions for all molecular markers were 95 °C/min followed by 28 cycles at 95 °C/15 s, 50 °C/45 s, and 60 °C/4 min. The generated sequences were assembled into contigs in BioEdit v.7.2.5 [30] and compared to homologous sequences deposited in the NCBI-GenBank database using BLAST. Data sets comprising the assembled sequences (those generated in this study and the sequences obtained from the database) were aligned in MAFFT v 7 [31]. The alignments were prepared separately for each fungus genus. Phylogenetic trees were inferred in MEGA v.7.1 [32], using the Neighbor-joining method and with Kimura 2-parameters nucleotide substitution model [33]. The robustness of the trees was calculated using the bootstrap method, with 1000 generations.

Accession Numbers

Sequences generated in this study were deposited in Genbank under accession numbers MG735773-MG736057 (yeasts) and MH128164-MH128317 (filamentous fungi).

Soil/Marine Sediment-Taxonomy Correlation, Species Diversity, Richness, and Distribution

Soil and marine sediment physical-chemical characteristics were related to the species composition profile of each sample by non-metric multidimensional scaling (NMDS). An analysis of similarities (ANOSIM) using a Bray-Curtis dissimilarity matrix was performed to verify if there were differences between soil sample groups (*C. quitensis* root-associated soil and *D. antarctica* root-associated soil) and between the two isolation temperatures. All analyses were performed using PAST v. 2.17c [34]. To quantify species diversity, the Shannon and the inverse Simpson indices were calculated and for species richness, the Chao-1 estimator was used. The similarities among fungal taxa from different samples were estimated using the Bray-Curtis, Sorensen, and Jaccard indices. All indices and estimators were calculated in EstimateS v.9.1.0 [35].

Screening of Cold-Adapted Enzymes

Soil isolates were screened for ligninolytic enzymes laccase, manganese peroxidase, and lignin peroxidase. The first screening was done on B&K medium with guaiacol 4 mM (during the isolation step) according to Verma et al. [36]. The presence of an intense brown color under the mycelium and surrounding it indicated the probable presence of laccase activity. Isolates that showed this characteristic were selected for the quantitative tests. Marine sediment isolates were screened for lipase and protease. Screening for lipase was done according to Kouker and Jaeger [37], with modification on Tubaki medium (in g L^{-1}): 1 peptone, 0.5 yeast extract, 15 agar, 31.25 mL of olive oil and Rhodamine B solution 0.01% (*v*/*v*). Lipase production was detected by the presence of a degradation halo around the colonies. For protease screening, the fungi were inoculated on skim milk medium (in g L^{-1}): 20 skim milk, 20 agar, and 0.2 M pH 7 phosphate buffer. The presence of a clear degradation halo around the colonies indicated protease production.

Cold-Adapted Enzymes Activity Quantification

Soil fungi screened positive for ligninolytic enzymes were cultivated in MA2% medium. After their growth, two 5-mm cylinders from the margin of the isolates' colonies were transferred to 150-mL flasks containing 50 mL of malt broth 2%. Assays were incubated at 15 °C for 7 days at 150 rpm in duplicates. After this step, the liquid media were centrifuged at 10,000 rpm for 30 min at 4 °C. Enzymatic activity was measured in triplicate using the broth obtained. Quantification of lignin peroxidase followed the method adapted from Arora and Gill [38]. Manganese peroxidase was quantified following the method from Wariishi et al. [39], and the determination of laccase activity was measured by the method reported by Buswell et al. [40]. Lipase quantification was determined following the method reported by Yang et al. [41]. The protease activity was determined as described by Charney and Tomarelli [42]. For ligninolytic enzymes, one enzymatic unit (U) was defined as the amount of enzyme needed to oxidize 1 μmol of substrate per minute. One unit of lipase activity (U) was defined as the amount of enzyme capable of releasing 1 μmol of p-NPP per mL per min of reaction. One unit of protease (U) was defined as the amount of enzyme required to increase the absorbance by 0.01 under the conditions used.

Results

Fungi from Soil Samples

A total of 891 isolates was obtained comprehending 399 yeasts and 492 filamentous fungi. The yeasts were recovered

on MA2% ($n = 117$), B&K ($n = 107$), BSA ($n = 96$), and diluted MA2% ($n = 79$). For filamentous fungi, BSA ($n = 172$), B&K ($n = 142$), MA2% ($n = 90$), and diluted MA2% ($n = 88$). From the two soil samples, 141 isolates were recovered and from the four root-associated soil samples, 750. The temperature of 15 °C resulted in a higher number of isolates (58.5%), whereas 41.5% were recovered at 5 °C. The ANOSIM for the temperatures confirmed that there was a significant difference between both employed temperatures ($R = 0.479$; $p = 0.002$).

The morphotyping step resulted in 66 morphotypes at 15 °C, and 85 morphotypes at 5 °C. For the yeasts, 114 different fingerprints were obtained. Thus, from the total 891 fungal isolates, 667 were sequenced. They were identified in 35 different taxa within the phyla Ascomycota, Basidiomycota, and Mucoromycota (Table 2). Most of the filamentous fungi belonged to the Ascomycota, and most of the yeasts belonged to the Basidiomycota. Six fungal taxa (Ascomycota) presented low molecular similarities in comparison to sequences in the NCBI-GenBank database. Even after phylogenetic trees were built, their identification remained inconclusive and therefore they were classified as NIA (non-identified ascomycetes; their phylogenetic trees are available in the Supplementary Material, Figs. S1-S5). Among the yeasts, the most represented taxa were *Leucosporidium* sp. (44.4%), the NIA6 (21.4%), *Goffeauzyma* sp. (11.2%), and *Holtermanniella* sp. (10.8%). Among the filamentous fungi, *Pseudogymnoascus* was the dominant taxon (31.7%) after NIA2 (5.1%) and *Mortierella* sp. 1 (4.5%). In contrast, the taxa *Vishniacozyma* sp., *Cadophora* sp., *Cosmospora* sp.3, *Laetinaevia* sp., *Thelebolus* sp., *Mortierella* sp.2, and NIA3 were the least dominant, with an abundance of ≤ 1.2% (Table 2). The most dominant genera, *Leucosporidium* and *Pseudogymnoascus*, were also dominant in all types of samples. *Mortierella* sp.2 was found exclusively in the soil sample S2. *Cadophora* sp., *Oidiodendron* sp., and *Thelebolus* sp. occurred exclusively in the *D. antarctica* root-associated soil sample S5. On the other hand, *Cosmospora* sp.3, *Laetinaevia* sp., and *Purpureocillium* sp. occurred exclusively in the *C. quitensis* root-associated soil sample S6. There were no species that occurred exclusively according to the plant species (*C. quitensis* or *D. antarctica*). Some taxa were isolated only at 5 °C, such as NIA3, NIA5, *Cadophora* sp., and *Thelebolus* sp. By contrast, the taxa NIA1, NIA2, *Cosmospora* sp.2 and *Cosmospora* sp.3, *Laetinaevia* sp., *Penicillium* sp., *Pochonia* sp., *Purpureocillium* sp., *Trichoderma* sp., and *Mortierella* sp.2 were isolated only at 15 °C. According to Table 2, 22.9% of total identified isolates were recovered from sample S6, 21.4% from sample S5, 19% from sample S4, 18.4% from sample S3, 10.8% from sample S2, and 7.3% from sample S1.

Table 2 Fungal taxa from soil samples

Taxon	S1	S2	S3	S4	S5	S6	Total
Ascomycota							
ANI1	0	2	3	0	2	1	8
ANI2	0	0	4	4	6	11	25
ANI3	0	0	1	0	0	0	1
ANI4	0	0	2	2	4	3	11
ANI5	0	0	2	0	1	1	4
ANI6	3	2	12	23	18	11	69
Antarctomyces sp.	1	0	11	2	2	3	19
Cadophora sp.	0	0	0	0	1	0	1
Cladosporium sp.	0	1	0	0	6	1	8
Cosmospora sp1	0	0	1	1	1	6	9
Cosmospora sp2	0	0	0	0	3	5	8
Cosmospora sp3	0	0	0	0	0	1	1
Fusarium sp.	0	0	1	2	10	5	18
Laetinaevia sp.	0	0	0	0	0	1	1
Leptosphaeria sp.	0	1	0	1	0	12	14
Microdochium sp.	0	0	1	6	1	3	11
Oidiodendron sp.	0	0	0	0	2	0	2
Penicillium sp.	1	0	2	1	1	0	5
Pochonia sp.	0	0	0	0	1	1	2
Pseudogymnoascus sp1	4	9	25	7	15	6	66
Pseudogymnoascus sp2	0	0	1	4	2	3	10
Pseudogymnoascus sp3	1	13	17	19	13	17	80
Purpureocillium sp.	0	0	0	0	0	3	3
Thelebolus sp.	0	0	0	0	1	0	1
Trichoderma sp.	0	0	0	0	7	3	10
Varicosporium sp.	0	0	2	2	0	0	4
Basidiomycota							
Cystobasidium sp.	0	0	3	0	3	4	10
Goffeauzyma sp.	1	4	6	21	1	3	36
Holtermanniella sp.	1	5	5	5	12	7	35
Leucosporidium sp.	29	23	20	23	17	31	143
Mrakia sp.	0	0	2	3	2	6	13
Naganishia sp.	7	4	1	1	0	1	14
Vishniacozyma sp.	0	0	1	0	1	0	2
Mucoromycota							
Mortierella sp.1	1	7	0	0	10	4	22
Mortierella sp.2	0	1	0	0	0	0	1
Total	49	72	123	127	143	153	667

S1 Yellow Point (yellowish soil), *S2* Yellow Point (dark brown soil), *S3* Punta Hennequin (*C. quitensis* root-associated soil), *S4* Punta Hennequin (*D. antarctica* root-associated soil), *S5* Punta Plaza (*D. antarctica* root-associated soil), *S6* Punta Plaza (*C. quitensis* root-associated soil)

Fungi from Marine Sediment Samples

A total of 226 isolates was obtained. In the first phase (with enrichment), 146 yeasts and 24 filamentous fungi were recovered from the five samples. The yeast isolates were recovered

with PDA ($n = 52$), diluted PDA ($n = 49$), BSA ($n = 23$), and MA ($n = 22$). For filamentous fungi, PDA ($n = 12$), PDA diluted ($n = 4$), BSA ($n = 8$), and no fungi were isolated from MA medium. From the second isolation phase (without enrichment), 56 isolates were recovered. A total of 20 yeasts was recovered from the four media: PDA ($n = 5$), PDA diluted ($n = 8$), MA ($n = 7$), and no isolate from BSA. Thirty-six filamentous fungi were recovered: PDA ($n = 4$), PDA diluted ($n = 5$), BSA ($n = 8$), and MA ($n = 19$). From the total of isolates, 68% were isolated at 15 °C. The ANOSIM for the temperatures confirmed that there was a significant difference between both employed temperatures ($R = 0.33$; $p = 0.03$).

According to sequencing data and phylogenetic analyses, representatives of nine yeast genera were recovered from the sediment samples (Table 3). The genus *Metschnikowia* was the most abundant (45.1%), followed by isolates from the genera *Mrakia* (21.6%), *Cryptococcus* and *Glaciozyma* (7.8%), *Meyerozyma* and *Holtermanniella* (5.4%), *Rhodotorula* (4.2%), *Cystobasidium* (1.8%), and *Phenoliferia* (0.6%). For filamentous fungi, representatives of eight genera were isolated from the marine sediment samples (Table 3). The genera *Penicillium* and *Pseudogymnoascus* were the most abundant (40.0%), followed by the genera *Cadophora* (6.6%), *Cladosporium* (5.0%), *Toxicocladosporium*, *Pseudocercosporella*, *Pestalotiopsis*, and *Paraconiothyrium* (1.6%). Some of the fungi identified in the present study have never been reported in Antarctic environments before (*Toxicocladosporium*, *Pseudocercosporella*, and *Paraconiothyrium*) and one isolate was not identified. According to Table 3, 35.8% of total isolates were recovered from sample Sed5, 24.7% from sample Sed4, 17.2% from sample Sed3, 11.5% from sample Sed1, and 10.6% from sample Sed2.

Although different culture conditions were applied to isolate fungi from terrestrial and marine samples, they shared the taxa *Pseudogymnoascus*, *Penicillium*, *Cladosporium*, *Cadophora*, *Mrakia*, *Goffeauzyma*, *Cystobasidium*, and *Holtermanniella* (Fig. 2).

Table 3 Fungal taxa from sediment samples

Taxon	Sed1	Sed2	Sed3	Sed4	Sed5	Total
Ascomycota						
Pseudogymnoascus sp.1	1	0	1	0	4	6
Pseudogymnoascus sp.2	0	0	0	1	0	1
Pseudogymnoascus sp.3	6	2	2	1	4	15
Pseudogymnoascus sp.4	0	0	0	2	0	2
Penicillium sp.	5	3	9	5	2	24
Cladosporium sp.1	0	1	0	0	0	1
Cladosporium sp.2	0	0	1	0	0	1
Cladosporium sp.3	0	0	1	0	0	1
Pestalotiopsis sp.	0	0	0	0	1	1
Pseudocercosporella sp.	0	0	0	1	0	1
Paraconiothyrium sp.	0	0	0	1	0	1
Toxicocladosporium sp.	0	0	0	1	0	1
Cadophora sp.	0	4	0	0	0	4
Metschnikowia sp.	1	6	2	22	44	75
Meyerozyma sp.	7	0	1	0	1	9
Non identified	0	0	1	0	0	1
Basidiomycota						
Rhodotorula sp.	0	0	0	1	6	7
Mrakia sp.	3	6	10	16	1	36
Cryptococcus sp.	0	0	11	1	1	13
Glaciozyma sp.1	0	2	0	1	3	6
Glaciozyma sp.2	1	0	0	0	0	1
Glaciozyma sp.3	2	0	0	0	4	6
Cystobasidium sp.	0	0	0	3	0	3
Holtermanniella sp.	0	0	0	0	9	9
Phenoliferia sp.	0	0	0	0	1	1
Total	26	24	39	56	81	226

Sed1 Botany Point, *Sed2* EACF, *Sed3* Punta Ullman, *Sed4* Refúgio 2 (Sediment), *Sed5* Refugio 2 (intertidal zone)

Soil–Taxonomy Correlation, Species Diversity, Richness, and Distribution

The NMDS analysis (Fig. 3a) revealed that the samples were separated by sampling location by coordinate 1 (samples S3 and S4 – Punta Hennequin, samples S5 and S6 – Punta Plaza). Samples S1 and S2, despite collected at the same place, they presented distinct physical and chemical characteristics, except for the carbon content, and appear distant from each other on the graph (Fig. 3a). The pH and the Fe and Al amounts are the characteristics that contribute to this distance. Data from soil physical and chemical characterizations are available in the Supplementary Information (Tables S1 and S2). The main factors that grouped samples S5 and S6 were the carbon content and the amounts of Fe and Al (Piro). There was no separation of samples by type (e.g., samples S3 and S6, *C. quitensis* root-associated soil, did not form a group) but by sampling location. The ANOSIM for the groups of samples *C. quitensis* root-associated soil and *D. antarctica* root-associated soil showed that there was no significant difference between the two groups ($R = -1$; $p = 1$). This means that the type of plant is not significant for the composition of the fungal communities.

The indices and richness estimator are listed in Table 4. Estimator Chao 1 showed that sample S6 has the major estimated richness. Shannon and Inverse Simpson indices were higher in samples S5 and S6. Species sharing analysis showed that the highest similarity occurred between samples S3 and S4 (68%) followed by samples S3 and S5 (63.9%) and S5 and S6 (63.5%). Samples that shared the least species were samples S1 and S5, with 31.3% of shared species, and samples S1 and S3, with 38.4% of species similarity.

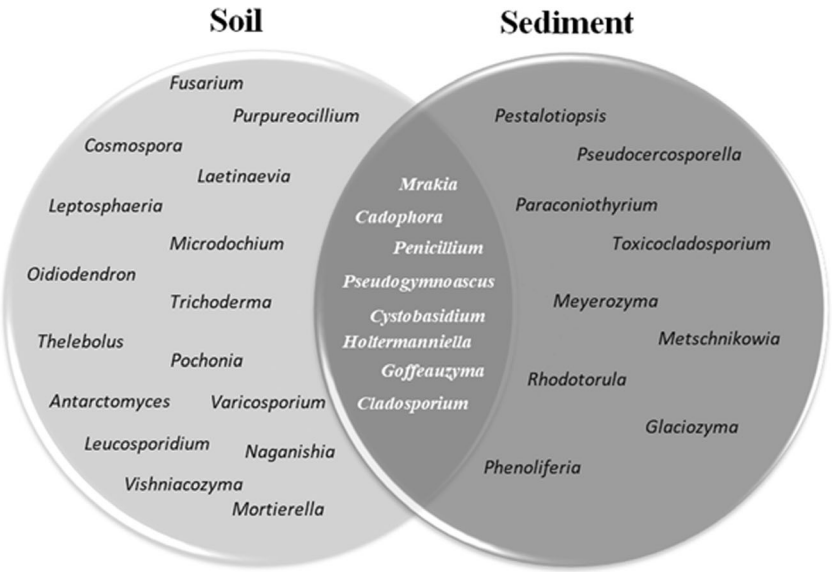

Fig. 2 Diagram showing the genera isolated from each type of sample

Marine Sediment–Taxonomy Correlation, Species Diversity, Richness, and Distribution

The NMDS analysis revealed that samples do not form a cohesive group since each sample is in a different place in the graph (Fig. 3b). All samples were collected at different places in Antarctica and showed unique characteristics. Data from sediment physical and chemical characterizations are available in the Supplementary Information (Tables S1 and S2). Samples Sed1, Sed2, and Sed3 are separated from Sed4 and Sed5. Besides this, coordinate 2 separated samples Sed3 and Sed4 from the others. Estimator Chao 1 showed that sample Sed4 has the highest estimated richness (Table 5); this sample has more rare species (singletons and doubletons) than the others. Shannon index was higher in sample Sed1, while Inverse Simpson was higher in sample Sed2 (Table 5). Species sharing (β-Diversity) showed that the major similarity occurred between samples Sed4 and Sed5 (42.3%), followed by samples Sed3 and Sed4 (40.0%). The least similarity occurred between samples Sed3 and Sed5 (only 15%).

Screening of Cold-Adapted Enzymes and Activity Quantification

From the 249 soil isolates grown on B&K, 35 showed probable laccase activity. Only one was from a soil sample, the others were from root-associated soil samples. Two isolates were recovered at 5 °C and the rest at 15 °C. Three isolates showed enzymatic activity higher than or equal to 1 U L^{-1} for lignin peroxidase: isolate 9P-1.9 (5.41 U L^{-1}), isolate 12P-3.10B (1.7 U L^{-1}), and 9P-3.19 (1 U L^{-1}). There was no detectable activity for manganese peroxidase and values for laccase were under 0.2 U L^{-1}. The 170 isolates (marine sediment) from the first isolation phase were tested for lipase production. In the qualitative step, 60 yeasts and 19 fungi showed a positive result. After this, the positive ones were tested in quantification phase, and 16 yeasts and nine fungi showed measurable lipase activity. Nevertheless, only nine yeast isolates were able to produce lipase above 0.5 U mL^{-1}. The best result (0.88 U mL^{-1}) was achieved by *Metschnikowia* sp. CRM1589. After the experimental design, this isolate increased its lipase production to 1.05 U mL^{-1} (data not shown). All the isolates from marine sediment were also tested for protease production. In the qualitative step, three yeasts and six filamentous fungi showed a positive result. After this, the positive isolates were tested in quantification phase, and all of them showed measurable protease activity. In this case, six filamentous fungi were able to produce protease above 2.0 U mL^{-1}. The best result (6.21 U mL^{-1}) was from *Pseudogymnoascus* sp. CRM1533. After the experimental design, the isolate *Pseudogymnoascus* sp. CRM1533 increased its protease production to 11.47 U mL^{-1} (data not shown).

Discussion

Fungal Isolation and Identification

The number of isolates and the different genera identified in this study revealed that yeasts and filamentous fungi could be recovered from extreme-condition samples of the Antarctic terrestrial and marine environments. As expected, there were more isolates retrieved from soil samples than from marine sediment, and more from root-associated soil than from bulk soil. As the roots release organic carbon, it is reasonable to believe that more isolates can be found in this environment [43]. Moreover, according to Berríos et al. [44],

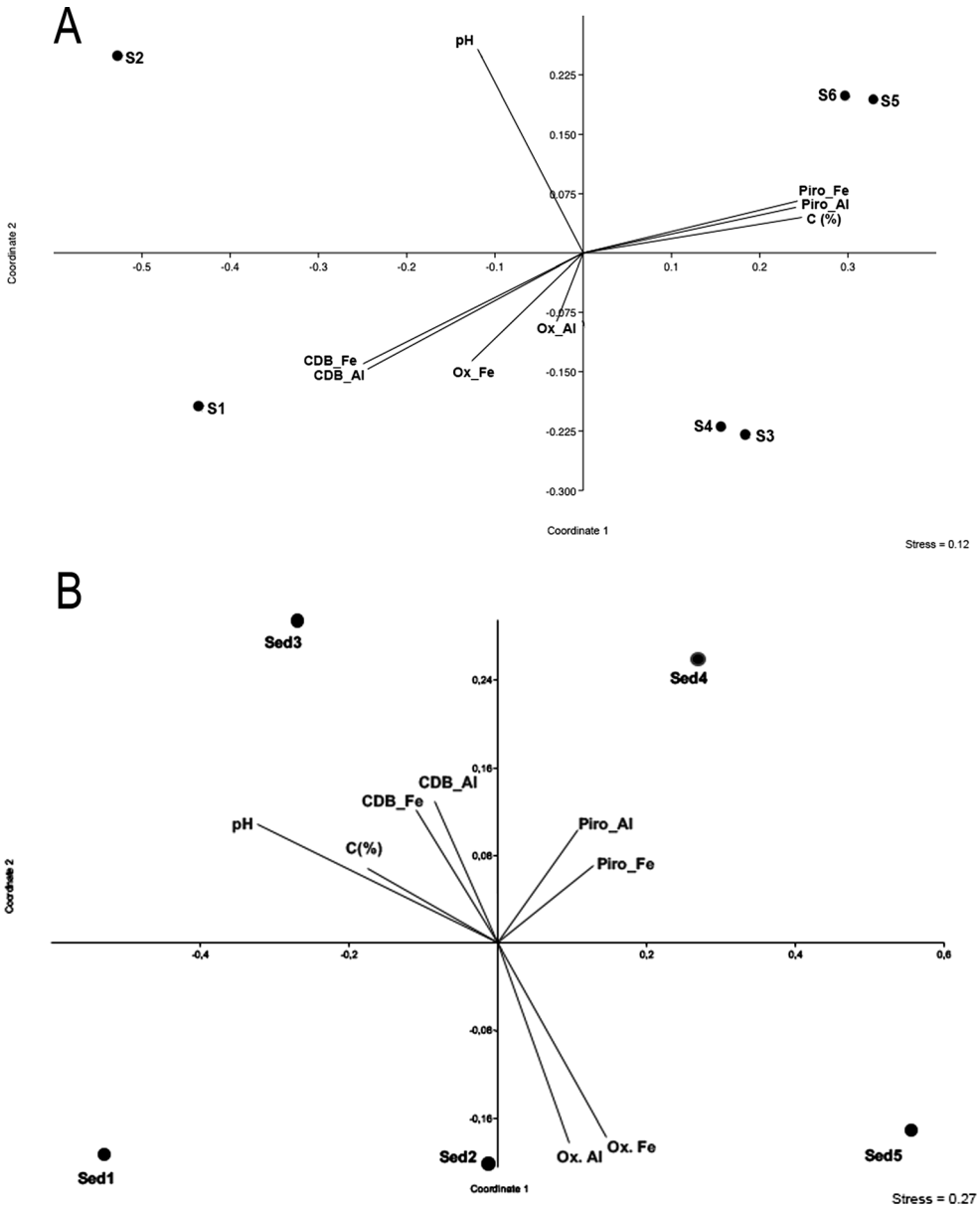

Fig. 3 Two non-metric multidimensional scaling (NMDS) biplots based on Bray-Curtis distances displaying the total fungal community distribution. **a** Soil samples and **b** marine sediment samples

some microorganisms from the rhizosphere of *D. antarctica* can have a significant role in the survival and adaptation of this plant in maritime Antarctica.

Ascomycota is the most common fungal phylum found in Antarctica [45]. In our study, we also had most of the filamentous fungi as representatives of this phylum. On the other hand, most of the yeasts were Basidiomycota. *Metschnikowia* was the most abundant yeast genus found in marine sediment samples, while *Leucosporidium* was the most abundant in soil samples. Representatives of the genus *Metschnikowia* are very common in Antarctica and also reported from water, ice, algae, sediments, and soil [28, 46–48]. On the other hand, *Leucosporidium* is widespread in alpine and polar environments [49] and was isolated from different substrates. The genera *Penicillium* and *Pseudogymnoascus* were the most abundant in marine sediments. In the soil samples, the most abundant ones were *Mortierella* and *Pseudogymnoascus*. According to Hayes [50], species from the genus *Pseudogymnoascus* are distributed globally and are common in cold environments. Moreover, Arenz et al. [51] suggest that this genus takes part in the decomposition and nutrient cycling in Antarctica. The genera *Penicillium* and *Pseudogymnoascus* are spread worldwide, and several species can develop in different and extreme conditions [50, 52].

An interesting result is the presence of *Toxicocladosporium*, *Pseudocercosporella*, and *Paraconiothyrium* in marine sediment samples. These genera have not been reported in the Antarctic environment yet. *Toxicocladosporium* is related to *Cladosporium* but now is recognized as a different genus [53]. The isolates in this study showed high sequence

Table 4 Indices and richness estimator (α and β-diversity) for soil samples

Sample	Shannon	Simpson Inv.	Chao1	S
S1 (Soil1)	1.44	2.61	24.69	10
S2 (Soil2)	2.04	5.79	12.99	12
S3 (Soil3)	2.51	8.89	25.47	22
S4 (Soil4)	2.35	7.94	19.19	18
S5 (Soil5)	2.84	13.32	32.96	27
S6 (Soil6)	2.8	11.51	46.86	26
First sample	Second sample	Jaccard	Sorensen	Bray-Curtis
S1	S2	0.571	0.727	0.612
S1	S3	0.391	0.563	0.384
S1	S4	0.474	0.643	0.409
S1	S5	0.321	0.486	0.313
S1	S6	0.321	0.486	0.416
S2	S3	0.308	0.471	0.574
S2	S4	0.364	0.533	0.563
S2	S5	0.3	0.462	0.53
S2	S6	0.393	0.564	0.533
S3	S4	0.739	0.85	0.68
S3	S5	0.633	0.776	0.639
S3	S6	0.581	0.735	0.601
S4	S5	0.5	0.667	0.578
S4	S6	0.552	0.711	0.621
S5	S6	0.688	0.815	0.635

similarity with *Toxicocladosporium strelitziae*; however, the phylogenetic tree did not confirm this identification. This genus is commonly associated with plants, and a large number of species are phytopathogens. Also, some species were recovered from clinic samples [53–57]. The genus *Pseudocercosporella* has several species that are associated with plants and also as phytopathogens [58]. The isolates showed 99% identity similarity with *Pseudocercosporella fraxini*, but the phylogenetic tree did not confirm this data. Most species of the genus *Paraconiothyrium* are usually associated with plants. This genus has been previously found in marine sponges and sea sediments [59–63].

Table 5 Indices and richness estimator (α and β-diversity) for sediment samples

Sample	Shannon	Simpson Inv.	Chao1	S
Sed1	1.83	5.37	9.44	8
Sed2	1.8	5.43	7	7
Sed3	1.82	4.83	13.25	10
Sed4	1.79	3.99	26.75	13
Sed5	1.73	3.11	21.41	14
First sample	Second sample	Jaccard	Sorensen	Bray-Curtis
Sed1	Sed2	0.364	0.533	0.36
Sed1	Sed3	0.5	0.667	0.4
Sed1	Sed4	0.313	0.476	0.268
Sed1	Sed5	0.467	0.636	0.206
Sed2	Sed3	0.308	0.471	0.413
Sed2	Sed4	0.25	0.4	0.4
Sed2	Sed5	0.313	0.476	0.248
Sed3	Sed4	0.278	0.435	0.4
Sed3	Sed5	0.333	0.5	0.15
Sed4	Sed5	0.35	0.519	0.423

Putative New Species

Among the total soil fungi recovered, six taxa were not identified at the genus level and were therefore labeled as "non-identified ascomycete" (NIA); NIA1 showed close relatedness to the only two described species for the genus *Rhizoscyphus*, which are commonly associated with the roots of *C. quitensis* and *D. Antarctica* [64]. The non-identified ascomycetes 2 and 3 had low bootstrap values in the phylogenetic tree, and therefore their identification remained inconclusive. NIA5 showed 93% similarity with *Urceolella carestiana*; in the phylogenetic tree, the isolate was placed very distantly from that species and could represent a new genus. Isolates identified as *Oidiodendron* sp. could also represent new species to science (for the phylogenetic tree, see Supplementary Material, Fig. S6). Among the marine isolates obtained in this study, one taxon was not identified due to its low similarity to other sequences in Genbank (for the phylogenetic tree, see Supplementary Material, Fig. S7). The best match was with *Phaeoacremonium santali*, but the similarity was only 86%.

Soil Diversity

There are few studies addressing fungi in root-associated soil in the two vascular plants that inhabit Antarctica. Vaz et al. [10] reported 12 different yeast taxa from the rhizosphere of *D. antarctica*. Gonçalves et al. [65] obtained nine different genera of filamentous fungi from the rhizosphere of *D. antarctica*. Microbial communities in root-associated soils are shaped by the plant species, and consequently by the type of exudates that they release [66, 67]. Thus, it was expected that *C. quitensis* and *D. antarctica* would present some influence on the fungal communities on their root-associated soils. Conversely, it was observed that the structure of the community was similar for samples collected in the same place, regardless of the plant species, and more related to the characteristics of the soil. Although there was no significant difference between the microbial composition found in root-associated soils of *C. quitensis* and *D. antarctica*, the fungal communities varied in the presence of plants among the sampling sites. The α-diversity analyses showed that samples S5 and S6 (*C. quitensis* and *D. antarctica* root-associated soil – Punta Plaza) were the richest and most diverse ones. The β-diversity results were expected since samples collected at the

same sampling point showed high rates of shared species. Moreover, root-associated samples showed higher species sharing among them.

According to Teixeira et al. [68], the presence of vascular plants in soils of Admiralty Bay plays an important role in the structure of bacterial communities. Possible explanations for this are the rhizosphere effect and the soil properties that influence more the structure of fungal communities. For example, the carbon content found in root-associated soil samples was higher than in soil samples (except for sample S4), and as the soil microbiota is composed mainly of heterotrophic microorganisms they rely on the carbon released by the roots as an energy source [67]. Another environmental factor related to the composition of the fungal communities was the temperature. There was a higher number of isolates obtained at 15 °C than at 5 °C, and the analysis of similarities showed that there was a significant difference between the communities' composition at these temperatures. Nonetheless, the test showed that the temperature itself is not a factor that fully explains the observed differences between the groups of isolates at 15 and 5 °C ($R = 0.479$), and other factors need to be taken into account. Studying soil communities along a transect of many islands in the Antarctic Peninsula, Dennis et al. [69] verified that the fungal communities' composition was not associated with latitude, which suggested that temperature is not a key factor in the composition of fungal soil communities along the Antarctic Peninsula. Comparing our results with the results from Dennis et al., it can be inferred that temperature plays a significant role in the local level (given that the sampling points in our study are placed on the same island and around the same bay), and once one analyzes soil communities on a larger scale, this factor becomes less significant.

Marine Sediment Diversity

The α-diversity analyses showed that sample Sed4 was the richest one, while Sed1 was the most diverse. The triplicates from sample Sed4 were collected at 20 m (average depth) and at 0.1 °C (average temperature), and triplicates from sample Sed1 were collected at 24.7 (average depth) and 0.6 °C (average temperature). These results revealed that samples collected at 20 m or deeper and at temperatures near 0 °C presented higher richness and diversity than the sample collected in the intertidal zone (superficial sample).

The results from β-diversity were expected; samples Sed4 and Sed5 showed higher species sharing. Both were collected in the same region. However, despite the higher similarity of these samples, the sharing percentage was low, below 50%. This hypothesis is sustained by the NMDS graph (where the samples were totally separated). Analyzing the distribution pattern and the isolated species, it is possible to infer that the geographic location is the main reason for the differences.

Samples Sed4 and Sed5 are distant from the others, probably because geographically their distance is higher. And the difference between Sed4 and Sed5 is probably due to the different types of the samples and their temperatures; Sed4 is from sediment and Sed5 from the intertidal zone. By bringing together all this information, it is possible to infer that fungal diversity in all samples changes due to the collecting region. Each sample is unique, and this provides the development of distinct fungal communities.

The ANOSIM showed that there was also a significant difference between the communities' composition at 15 and 5 °C. As for the soil samples, temperature itself is not a factor that fully explains the observed differences between the groups of isolates at the two temperatures ($R = 0.33$).

Cold-Adapted Enzymes

Ligninolytic Enzymes

The majority of filamentous fungi positive for lignignolytic enzymes in the first screening were isolated at 15 °C and come from root-associated soil. The low amount of fungi capable of producing ligninolytic enzymes could be related to the restriction of lignocellulosic material in Antarctic environments. Although no laccase activity was observed for yeasts, Vishniac [70] reported that lineages of *Cryptococcus* isolated from Antarctica could produce laccase, among other enzymes. In a previous study from our group, with 160 filamentous fungi isolated from different Antarctic substrates (wood, sea stars, marine sediment, lichen, algae), it was verified that 29 had probable laccase activity using the same screening method as used here (data not publishd yet). As far as we know, there is no study related to the production of ligninolytic enzymes by Antarctic filamentous fungi.

Lipase and Protease

Data from lipase in solid and liquid media revealed that yeasts were more expressive in lipase production than the filamentous fungi. Among the positive yeasts, 56.2% were recovered from sample Sed5 (Refugio 2 - intertidal zone). This result can be justified by the fact that this sample came from a transition zone, where the accumulation of oil and fat (from dead animals) and pollution (oil, boat fuel) brought by tides is more likely to happen. Duarte [71] reported lipase activity from two genera isolated from Antartic samples: *Cryptococcus* and *Leucosporidium* (between 0.1 and 0.23 U mL^{-1}). In this study, the highest activity (0.88 U mL^{-1}) was from *Metschnikowia* sp. CRM 1589. Yeasts from the phylum Ascomycota, in general, are able to produce lipase, especially the ones from the genera *Candida*, *Yarrowia*, and *Saccharomyces* [72]. Vaca et al. [73] reported lipase activity from two isolates of *M. australis*, but only the qualitative procedure was done.

Protease activity was more expressive in filamentous fungi than in yeasts. All positive isolates were recovered from sediment samples (Sed1, Sed2, Sed3 e, Sed4) and none from the intertidal zone (Sed5). *Pseudogymnoascus* sp. CRM1533 showed higher protease activity (6.21 U mL^{-1}). The protease production ability of the *Pseudogymnoascus* genus has been reported in literature, but most of the studies only do the qualitative screening, and there is not much data about protease activity quantification [74–77].

In conclusion, results from the present work revealed that *Pseudogymnoascus* sp. and representatives of the genus *Penicillium* were dominant in all marine sediment samples, while *Metschnikowia* was the most abundant yeast genus in this Antarctic environment. *Pseudogymnoascus* sp. was also dominant in soil samples, as well as yeasts of the genus *Leucosporidium*. We showed that despite being located within the same bay, each sampling point we assessed is unique in its physical-chemical composition and fungal community. This shows the complexity of the Antarctic environment and that further studies need to be performed to fully understand the dynamics of fungal communities. Oxidative and hydrolytic enzymes were produced by fungal isolates from soils and marine sediments, respectively. Although few soil fungi presented the capacity to produce ligninolytic enzymes, many fungi were able to produce protease and lipase. The fungal collection obtained in the present study is currently being investigated in the search for plant growth promoter and anticancer compounds and for biopesticides.

Funding Information This paper was supported by grants financed by FAPESP (reference numbers: #2013/19486-0 and #2016/07957-7), and by scholarships financed by CAPES. LDS and AR thank the National Council for Scientific and Technological Development (CNPq) for Productivity Fellowships 304103/2013-6 and 305341/2015-4. LDS thanks MICROSFERA project (PROANTAR/CNPq) for the support with sample collection.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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