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ORIGINAL PAPER

Production of cold-adapted enzymes by filamentous fungi from King George Island, Antarctica

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Abstract

Antarctic environments are characterized by polar climate, making it difficult for the development of any form of life. The biogeochemical cycles and food web in such restrictive environments may be exclusively formed by microorganisms. Polar mycological studies are recent and there is much to know about the diversity and genetic resources of these microorganisms. In this sense, the molecular taxonomic approach was applied to identify 129 fungal isolates from marine and terrestrial samples collected from the King George Island (South Shetland Islands, Maritime Antarctic). Additionally, the production of cold-adapted enzymes by these microorganisms was evaluated. Among the 129 isolates, 69.0% were identified by ITS-sequencing and affiliated into 12 genera. *Cadophora, Geomyces*, *Penicillium*, *Cosmospora*, and *Cladosporium* were the most abundant genera. Representatives of *Cosmospora* were isolated only from terrestrial samples, while representatives of the others genera were recovered from marine and terrestrial samples. A total of 29, 19, and 74 isolates were able to produce ligninolytic enzymes, xylanase, and L-asparaginase, respectively. Representatives of *Cadophora* showed great ability to produce lignin peroxidase (LiP) and laccase at 15.0 °C in liquid medium, while representatives of *Penicillium* and non-identified fungi were the best producers of xylanase and L-asparaginase at 20.0 °C. The high number of fungi able to produce enzymes at moderate temperature reveals their potential for industrial production and biotechnological applications. The present study broadens the knowledge of fungal diversity associated with the southern polar region. Additionally, data from molecular taxonomy suggest that two filamentous fungi may be considered as potential new species.

Keywords Extremophiles · Microbial biotechnology · Ligninolytic enzymes · L-Asparaginase · Xylanase

Introduction

The Antarctic environment, including the maritime and continental areas, is considered the coldest, driest, and most inhospitable place on Earth, covered by ice and surrounded by frozen sea (Tindall 2004). As a restrictive environment, diversity in general tends to be lower, and in some systems, biogeochemical cycles and food chains are exclusively formed by microorganisms (Vincent 2000).

The majority of the filamentous fungi that occur in the Antarctic environments are found on other continents, while few species are known to be endemic. Considering the growth temperature, fungi that live in these environments are classified as psychrophilic, psychrotolerant (psychrotrophic), and mesophilic-psychrotolerant. The first ones are able to grow at 0.0 °C with optimum growth at temperatures ≤ 15.0 °C, and maximum growth at temperatures ≤ 20.0 °C. Psychrotolerant fungi can grow at 0.0 °C,

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but their maximum growth temperature is between 15.0 and 25.0 °C, while the mesophilic-psychrotolerant have the ability to grow at low temperatures, with optimum growth at temperatures > 25.0 and ≤ 40.0 °C (Ruisi et al. 2007; Pesciaroli et al. 2012). According to Godinho et al. (2013), most of the filamentous fungi recovered from Antarctic samples are psychrotolerant, and few are psychrophilic.

The production of enzymes active at low temperatures is one of several survival strategies developed by microbial communities that inhabit Antarctica (Loperena et al. 2012; Duarte et al. 2018). Cold-adapted enzymes have properties that could be interesting for many industrial and/or environmental sectors (Siddiqui and Cavicchioli 2006). According to Feller and Gerday (2003), cold-adapted enzymes are up to tenfold more active at low/moderate temperatures than their mesophilic homologs. Additionally, they can be inactivated by temperature before the unfolding of the protein structure and their heat-lability (mainly in psychrophilic and psychrotolerant microorganisms) can propitiate selective inactivation in complex mixtures. In this context, in the present study, we investigated the production of ligninases, xylanases, and L-asparaginases by filamentous fungi from the Antarctic.

Cold-adapted ligninases and xylanases can be applied in the degradation of lignocellulosic material for biofuel production and in bioremediation of cold environments or processes (Margesin et al. 2002; Del-Cid et al. 2014). Cold-active xylanases are also useful for the generation of chemicals from lignocellulose (Del-Cid et al. 2014). Additionally, the ability of filamentous fungi from Antarctic environments to produce ligninases, xylanases, and/or L-ASNase at moderate temperatures can offer economic benefits by reducing energy costs in production processes conducted at moderate temperatures (25.0–40.0 °C) (Feller and Gerday 2003).

Considering the biotechnological potential of extremophile microorganisms from cold environments, in this study, we assessed filamentous fungi recovered from different samples collected on King George Island (Maritime Antarctica) and also evaluated their ability of producing of cold-adapted enzymes.

Materials and methods

Sampling and isolation

The fungal isolates ($n = 129$) were recovered from different marine and terrestrial substrates collected from the King George Island (Maritime Antarctica) by the Brazilian Antarctic Program team during an expedition in the southern polar summer of 2010. Sample description and GPS data are listed in Table 1.

Filamentous fungi were isolated on potato dextrose agar medium (in g L^{-1}: 200.0 potato extract, 20.0 dextrose, and 20.0 agar) supplemented with rifampicin (300.0 µg mL^{-1}) and incubated at 15.0 °C. Cultivation and purification of the isolates were performed on the same agar medium. All isolates are maintained in two different preservation methods (cryopreservation at − 80.0 °C in 10% glycerol and in water at 4.0 °C—Castellani) at the Brazilian Collection of

Table 1 Samples and collecting sites on King George Island, Antarctica

Samples	Site	Location (GPS)
Marine		
Isopod	Punta Plaza	62º05′S 58º24′W
Nacella sp.	Comandante Ferraz Antarctic Station	62º05′130″S 58º23′356″W
Sponge	Comandante Ferraz Antarctic Station	62º05′130″S 58º23′536″W
Ascidian	Comandante Ferraz Antarctic Station	62º05′130″S 58º23′536″W
Salpa sp.	Punta Plaza	62º05′S 58º24′W
Sea star	Comandante Ferraz Antarctic Station	62º05′130″S 58º23′536″W
Marine sediment	Punta Ulmann (21.6 m)	62º05′015″S 58º20′987″W
Marine sediment	Refúgio 2 (21.0 m)	62º04′373″S 58º25′335″W
Marine sediment	Botany Point (26.0 m)	62º05′734″S 58º19′919″W
Marine sediment	Comandante Ferraz Antarctic Station (22.8 m)	62º05′130″S 58º23′356″W
Macroalgae	Punta Plaza	62º05′S 58º24′W
Terrestrial		
Lichens	Punta Plaza	62º05′S 58º24′W
Woods	Punta Plaza	62º05′S 58º24′W
Whale bones	Punta Plaza	62º05′S 58º24′W
Ornithogenic soil	Demay Point Arctowski Station	62º12′S 58º25′W 62º08′S 58º27′W

Sampling was carried out in 2010

Environmental and Industrial Microorganisms—CBMAI (UNICAMP, SP, Brazil) and at the UNESP Central of Microbial Resources—CRM-UNESP (UNESP, SP, Brazil).

Taxonomic identification

Filamentous fungi identification was carried out by ITS barcode sequencing and phylogenetic analysis. The ITS1-5.8S-ITS2 region of the ribosomal DNA was amplified from the genomic DNA extracted from fungal isolates. DNA extraction was carried out following a combined protocol from Moller et al. (1992) and Gerardo et al. (2004) using freshly grown mycelia. ITS4 and ITS5 primers (White et al. 1990) were used for the amplification. PCR conditions were the following: initial denaturation at 94.0 °C for 3 min, followed by 35 cycles at 94.0 °C for 1 min, 55.0 °C for 1 min, and 72.0 °C for 2 min. Amplicons were cleaned up with the Wizard® SV Gel mini kit and PCR Clean-Up System (Promega) and quantified in NanoDrop® (Thermo Scientific). Cycle sequencing reactions were carried out using the BigDye Terminator v. 3.1 Cycle Sequencing Kit (Life Technologies) following the manufacturer's instructions. The products were sequenced on ABI 3330xl (Life Technologies). Forward and reverse sequences were compiled into contigs in BioEdit v. 7.0.5.3 (Hall 1999). Contigs were queried using the NCBI-GenBank (http://www.ncbi.nml.nih.gov) database to find the closest known sequences.

Sequences of the ITS region were aligned with sequences from the closest relatives found in the database in MAFFT v.7.110. (Katoh and Standley 2013) and manually inspected in Bioedit. The trees were inferred using MEGA v. 6.0 (Tamura et al. 2011) by the neighbor-joining algorithm, Kimura 2-parameters as the nucleotide substitution model, and 1000 bootstrap pseudo-replicates. Sites containing gaps were excluded from the analysis.

Screening of cold-adapted enzymes

Ligninolytic enzymes

The isolates were submitted to a screening on the solid medium using the method described by Verma et al. (2010). Therefore, fungal strains were cultivated at 15.0 °C in B&K medium containing (in g L^{-1}): 10.0 glucose, 2.0 peptone, 1.0 yeast extract, 20.0 agar, and 4.0 mM guaiacol. The presence of brown coloring (guaiacoquinone) on and around the mycelium in the medium supplemented with guaiacol indicated that ligninolytic enzymes are present.

Xylanase

Antarctic-derived fungi were inoculated in tubes containing 1.0 mL of YNB (Yeast Nitrogen Base) medium with xylan as the carbon source. The cultures were incubated at 20.0 °C and at 120 rpm for 7 days. After this period, the enzymatic broth (supernatant) was obtained by centrifugation (15 min at 3000 rpm). Aliquots of 25.0 µL were transferred to sterilized straws arranged into Petri dishes containing agar-xylan medium. Plates were incubated for 48 h at 20.0 °C. After the incubation time, the straws were removed and the culture medium was colored with lugol. The presence of a degradation halo in the culture medium indicated that enzymes are present (Costa 2014).

L-Asparaginase

Screening was performed using Czapek Dox medium (Gulati et al. 1997) containing (in g L^{-1}): 2.0 glucose, 10.0 L-asparagine, 1.52 KH_2PO_4, 0.52 KCl, 0.52 $MgSO_4{\cdot}7H_2O$, 0.001 $CuNO_3{\cdot}3H_2O$, 0.001 $ZnSO_4{\cdot}7H_2O$, and 0.001 $FeSO_4{\cdot}7H_2O$, pH 6.2, added with phenol red (0.009% final concentration). Plates were incubated for 7 days at 20.0 °C (L-ASNase activity was identified by the formation of a pink zone around colonies).

Assay of enzymatic activities

Ligninolytic enzymes

Fungal strains were cultivated in 2% (w/v) malt extract agar (MA2). Mycelium fragments (0.5 cm in diameter) taken from the edge of the colonies ($n = 3$) were transferred to 150-mL Erlenmeyer flasks containing 50 mL of MA2%. The assays were incubated at 15.0 °C and 25.0 °C under agitation at 150 rpm for 7 days, in duplicate. The extracts were obtained by centrifuging the liquid culture medium at 10,000 rpm for 30 min at 4.0 °C. Enzymatic activity was determined in triplicate using different enzyme substrates according to the methodology described below. One enzyme unit was defined as 1.0 µmol of product formed per minute under the assay conditions, obtained from calculations performed from Eq. 1, which was derived from the Beer–Lambert Law:

$$UL - 1 = \Delta A \times V \times 106/\varepsilon \times R \times T, \quad (1)$$

where ΔA is the difference between the final and initial absorbance, V is the reaction volume (0.001 L in all cases), 10^6 is the conversion of moles from ε to µmols, ε is the extinction coefficient (M^{-1} cm^{-1}), R is the amount of enzyme in the broth (L), and T is the reaction time (min).

Laccase activity

Laccase activity was determined using 2.2′-azinobis-(3-ethylbenzothiazoline (ABTS) as previously described by Buswell et al. (1995). The mixture was composed of 0.3 mL

sodium acetate buffer (0.1 M pH 5.0), 0.1 mL ABTS solution (0.03% w/v), and 0.6 mL enzyme solution. ABTS oxidation was measured by monitoring the increase in absorbance at 420 nm ($\varepsilon = 36{,}000\ M^{-1}\ cm^{-1}$) at 37.0 °C for 10 min.

Lignin peroxidase activity

LiP activity was determined by the oxidation of veratryl alcohol as previously described by Tien and Kirk (1984). The mixture reaction was composed of 500.0 µL enzyme extract, 1.0 mL sodium tartrate buffer (125.0 mM pH 3.0), 500.0 µL veratryl alcohol (10.0 mM), and 500.0 µL hydrogen peroxide (2.0 mM). The reaction was initiated with hydrogen peroxide and the appearance of veratraldehyde was measured at 310 nm ($\varepsilon = 9300\ M^{-1}cm^{-1}$).

Xylanase

Fungal strains were cultivated in 2% (w/v) malt extract agar (MA2) for 7 days at 20.0 °C. After this period, three fungal plugs (0.5 cm in diameter) taken from the edge of the colony were transferred to 200-mL Erlenmeyer flasks containing 50.0 mL of Mandels and Sternberg medium (MS), consisting of (in g L^{-1}) 1.0 peptone, 1.4 $(NH_4)_2SO_4$, 2.0 KH_2PO_4, 0.3 urea, 0.3 $CaCl_2$, 0.3 $MgSO_4{\cdot}7H_2O$, 0.005 $FeSO_4{\cdot}7H_2O$, 0.001 $MnSO_4{\cdot}H_2O$, 0.001 $ZnSO_4{\cdot}7H_2O$, and 0.002 $CoCl_2$, pH 6.0, with 10.0 birchwood xylan (Sigma-Aldrich) as the carbon source. Assays were incubated for 7 days at 140 rpm and 20.0 °C. Cultures were harvested by centrifugation at 12,074×*g* for 30 min and the supernatant was used for the enzymatic quantification.

Xylanase activity

Xylanase activity was quantified by determining the amount of reducing sugar released from xylan derived from birchwood according to Bailey et al. (1992). The xylanase activity assay was performed by adding 20.0 µL of enzymatic broth into 50.0 mM citrate buffer (pH 5.0) with 1% (w/v) birchwood xylan (Sigma) at 40.0 °C for 5 min. The generated reducing sugar was measured by using dinitrosalicylic acid (DNS) (Miller 1959). One unit of enzyme activity was defined as the amount of enzyme required to release 1 µmol of product equivalent per min in assay conditions.

L-Asparaginase

Fungal strains were cultivated in PDA at 20.0 °C for 7 days. After this period, three mycelium fragments (0.5 cm in diameter) taken from the edge of the colonies were transferred to 200 mL-Erlenmeyer flasks containing 50.0 mL of the modified Czapek Dox medium consisting of 10.0 g L^{-1} L-proline as the inducer of L-ASNase, and incubated at 20.0 °C for 7 days. Cultures were harvested by centrifugation at 12,074 g for 30 min and the supernatant was used for enzymatic quantification.

L-Asparaginase activity

L-ASNase activity was measured by the ammonia produced during the hydrolysis of L-asparagine (Sigma–Aldrich/USA) catalyzed by the enzyme through Nessler's reagent (Merck–Millipore). The method is divided into two steps. The first step was performed by adding 0.1 mL of enzymatic broth, 1.0 mL Tris buffer (50 mM) pH 8.6, asparagine 0.1 mL (189.0 mM), and 0.9 mL of Milli-Q water. The mixture was incubated at 37.0 °C for 30 min. After this period, 0.1 mL of trichloroacetic acid (TCA) 1.5 M was added to stop the reaction. In the second step, the mixture reaction was composed of 0.2 mL supernatant, 4.3 mL milli-Q water, and 0.5 mL Nessler's reagent. The reading was conducted spectrophotometrically at 436 nm. The concentration of ammonia produced in the reaction was determined based on a standard curve obtained using ammonium sulfate as standard (0.0 to 20.0 µmol mL^{-1}). One L-ASNase unit was considered as the amount of enzyme that catalyzes the formation of 1.0 µmol of ammonia per min at 37.0 °C and was expressed (Gulati et al. 1997; Theantana et al. 2007).

Results

Taxonomic evaluation

From the total number of isolates ($n = 129$), 89 (69%) were identified based on molecular taxonomy, the other fungal isolates (31%) could not be identified due to PCR amplification failures or low-quality sequences. Data derived from sequencing (Table 2) (Online Resource 1, Table S1) and phylogenetic analyses (Online Resource 1, Fig. S1) revealed that the Antarctic filamentous fungi recovered from the terrestrial and marine samples belong to 12 ascomycetous genera: *Acremonium*, *Cadophora*, *Cercospora*, *Cladosporium*, *Cosmospora*, *Geomyces*, *Hypocrea, Oidiodendron*, *Penicillium, Pseudeurotium*, *Pseudogymnoascus*, and *Thelebolus*.

Representatives of the genus *Cadophora* ($n = 29$, 32.5%) were isolated in greater abundance, followed by *Geomyces* ($n = 18$, 20.4%), *Penicillium* ($n = 12$, 13.6%), *Cosmospora* ($n = 8$, 9%), *Cladosporium* ($n = 7$, 9%), and the genera *Thelebolus, Oidiodendron, Acremonium, Cercospora, Hypocrea,* and *Pseudeurotium,* which were isolated in low abundances.

Fungal isolates representing the three most abundant genera were recovered from marine and terrestrial samples. Filamentous fungi affiliated to the *Cadophora* genus were recovered from wood ($n = 11$), marine sediment ($n = 5$), lichen ($n = 4$), ascidian ($n = 3$), sea star ($n = 2$), sea urchin

Table 2 Taxonomic affiliation of the Antarctic fungi isolated from samples collected from the King George Island (summer 2010)

Fungal identification[a]	BLAST closest relatives	ID (%)	Accession number	No. of isolates	Origin
Acremonium sp.	*Acremonium alternatum* CBS 233.70	100	AY566992	2	Lichen
Cadophora luteo-olivacea	*Cadophora luteo-olivacea* CBS 357.51	100	GU128589	11	Lichen, marine sediment, Wood
Cadophora malorum	*Cadophora malorum* CBS 687.96	100	GU128592	17	Ascidian, wood, urchin, sea star, *Salpa* sp., sediment
Cadophora sp.	*Cadophora melinii* CBS 268.33	100	NR111150	1	Whale bones
Cercospora sp.	*Cercospora ariminensis* CBS 137.56	100	KF251297	1	Urchin
Cladosporium halotolerans	*Cladosporium halotolerans* CBS 119416	100	NR_119605	1	Isopod
Cladosporium sp.	*Cladosporium cladosporioides* CBS 674.82	100	HM148014	6	Wood, amphipod, ornithogenic soil, *Salpa* sp., macroalgae
Cosmospora sp.	*Cosmospora viridescens* CBS 102433	99	KC291731	8	Ornithogenic soils, whale bones
Geomyces sp.	*Geomyces* sp. UFMGCB 5995	100	KC811057	18	Amphipod, isopod, sea star, lichen, wood, *Salpa* sp., *Nacella concinna.* and marine sediment
Hypocrea sp.	*Hypocrea pilulifera* CBS 120927	99	FJ860810	1	Lichen
Oidiodendron sp1	*Oidiodendron* sp.	97	JX270506	1	Macroalgae
Oidiodendron sp2	*Oidiodendron* sp.	100	JX270395	1	Lichen
Penicillium sp.	*Penicillium commune* CBS 311.48	100	AY213672	12	Sea star, lichen, wood, *Nacella concinna*, whale bones, *Salpa* sp., isopod
Pseudeurotium sp1	*Pseudeurotium desertorum* CBS 986.72	97	AY129288	1	Wood
Pseudeurotium sp2	*Pseudeurotium hygrophilum* CBS 102670	100	NR111128	3	Marine sediment
Pseudogymnoascus sp.	*Pseudogymnoascus* sp. UFMGCB1617	100	KJ183212	3	*Nacella concinna*, sea star, *Salpa* sp.
Thelebolus sp.	*Thelebolus globosus* CBS 113940	99	DQ028268	2	Star, ornithogenic soil
Total				89	

CBS Fungal Biodiversity Center (Utrecht, Netherlands), *UFMGCB* Collection of Microorganisms and Cells of UFMG (Universidade Federal de Minas Gerais, Belo Horizonte, Brazil)

[a]Final identification considering BLAST and phylogenetic analyses

($n=1$), *Salpa* sp. ($n=1$), and whale bones ($n=1$). Representatives of three different taxa of *Cadophora* were identified: *Cadophora malorum*, *Cadophora luteo-olivacea,* and *Cadophora* sp. The genus *Geomyces* was the second in abundance and was recovered from eight different samples (Table 2). The majority of isolates was recovered from lichen samples ($n=8$) and the other ones from *Salpa* sp. ($n=4$), amphipod ($n=2$), wood ($n=1$), isopod ($n=1$), sea star ($n=1$), and marine sediment ($n=1$). *Penicillium* isolates were recovered from seven different samples, including sea star ($n=3$), lichen ($n=2$), wood ($n=2$), *Nacella* sp. ($n=2$), and isopod, whale bones, and *Salpa* sp. ($n=1$, each one).

The genus *Cosmospora* was recovered only from terrestrial samples, including whale bones ($n=7$) and ornithogenic soil ($n=1$). Representatives of *Cladosporium* were isolated from marine and terrestrial samples, one isolate was recovered from isopod identified as *C. halotolerans*, and the others identified as *Cladosporium* sp. and recovered from ornithogenic soil ($n=2$), wood ($n=1$), amphipod ($n=1$), *Salpa* sp. ($n=1$), and macroalgae ($n=1$). Additional genera isolated in less abundance were *Thelebolus* (sea star and ornithogenic soil), *Oidiodendron* (lichen and macroalgae), *Acremonium* (lichen), *Cercospora* (sea urchin), *Hypocrea* (lichen), and *Pseudeurotium* (wood and marine sediment).

Cold-adapted enzymes

Ligninolytic enzymes

A total of 29 (22.4%) filamentous fungi were selected based on the presence of brown coloring in solid medium containing guaiacol, which indicates the presence of ligninolytic enzymes. Some fungi showed a very intense brown color around the mycelium, while others had a brown color a bit lighter, which may be associated with the enzymatic potential of each isolate.

Antarctic filamentous fungi considered positive for ligninolytic enzymes were isolated from terrestrial ($n=17$) and marine samples ($n=12$). The majority of ligninolytic fungi were recovered from wood ($n=13$), others were isolated from sea star ($n=3$), marine sediment ($n=2$), lichens ($n=2$), whale bones ($n=2$), macroalgae ($n=1$), amphipod ($n=1$), isopod ($n=1$), *Nacella* sp. ($n=1$), sea urchin ($n=1$), and *Salpa* sp. ($n=1$).

Most of the ligninolytic fungi were identified as belonging to the genus *Cadophora* ($n=12$), ten isolates were representatives of *Cadophora malorum* and two *Cadophora luteo-olivacea*. Other fungi selected based on the potential to produce ligninolytic enzymes were identified as representatives of the genera *Penicillium, Oidiodendron*, *Cosmospora, Geomyces,* and *Cladosporium.* A total of four fungal isolates could not be identified by molecular taxonomy.

Among the selected fungi ($n=29$), *Cadophora luteo-olivacea* P1 and *Cadophora malorum* A2B showed the highest laccase activity at 15.0 °C (Fig. 1a), both recovered from marine samples: marine sediment and sea star, respectively. *Cadophora malorum* AS-2A, also recovered from the marine sample (ascidian), produced the highest amount of laccase at 25.0 °C (Fig. 1a). Other isolates were able to produce laccase in very low amounts. On the other hand, the best production of LiP at 15.0 °C and 25.0 °C (Fig. 1b) was achieved by fungi recovered from terrestrial samples: *Cosmospora* sp. OB4B, which was recovered from whale bones and *Cadophora malorum* M7, recovered from the wood sample.

A higher number of fungi from marine origin were able to produce laccase and LiP at 25.0 °C in comparison to the number of isolates capable of producing these enzymes at 15.0 °C. On the other hand, a higher number of fungi recovered from terrestrial samples were able to produce these enzymes at 15.0 °C (Fig. 2).

Xylanase

A total of 19 (14.7%) Antarctic filamentous fungi were able to degrade xylan in the solid medium, seven were isolated from terrestrial samples, and 12 from marine samples: sea star ($n=3$), amphipod, *Nacella* sp. ($n=2$); marine sediment, snail, *Salpa* sp., ascidian ($n=1$), wood ($n=3$), lichens, whale bones, ornithogenic soil ($n=1$). Among the positive isolates, four were identified as *Penicillium* sp. and three as *Cadophora luteo-olivacea.*

The results of enzymatic activity revealed that seven filamentous fungi were able to produce more than 10 U mL^{-1} of xylanase (Fig. 3). Three of them were identified as *Penicillium* sp. (E2B, N5, and E2-1) and four could not be identified based on barcode sequences (N2C, C2, L1-4, and E2C). The highest number of fungi able to produce xylanase was recovered from marine sources. The unidentified fungus N2C (recovered from *Nacella* sp.) and *Penicillium* sp. E2B (recovered from sea star) showed the best xylanase activity.

L-asparaginase

A total of 74 (57%) Antarctic filamentous fungi were positive for L-ASNase based on the formation of a pink zone in solid medium as a result of the conversion of the phenol red from yellow (acidic condition) to pink (alkaline condition). Among the positive strains, 36 and 38 were isolated from terrestrial and marine samples, respectively: S*alpa* sp. ($n=12$), sea star ($n=8$), marine sediment ($n=7$), isopod

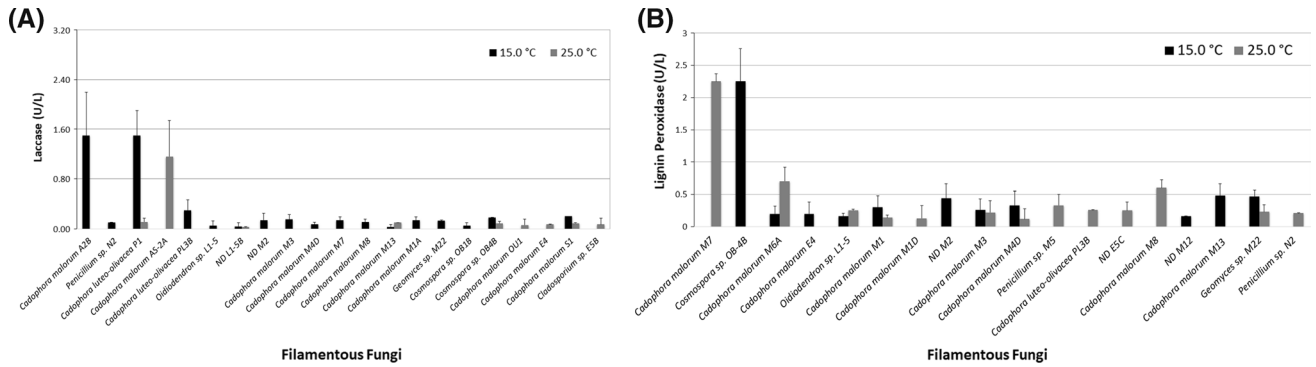

Fig. 1 Laccase (**a**) and LiP (**b**) production by Antarctic filamentous fungi after 7 days of cultivation at 15.0 °C and 25.0 °C and at 150 rpm

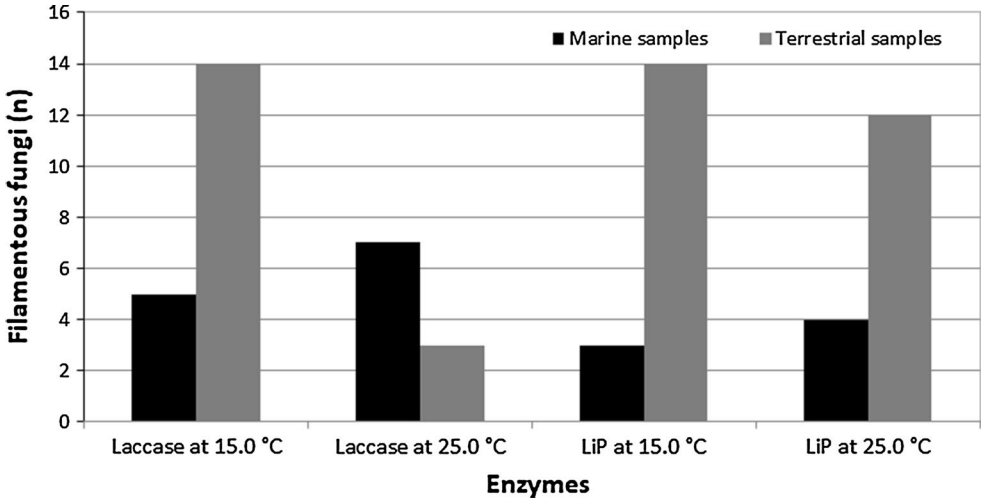

Fig. 2 Number of Antarctic fungal isolates from marine and terrestrial samples able to produce ligninolytic enzymes at 15.0 °C and 25.0 °C

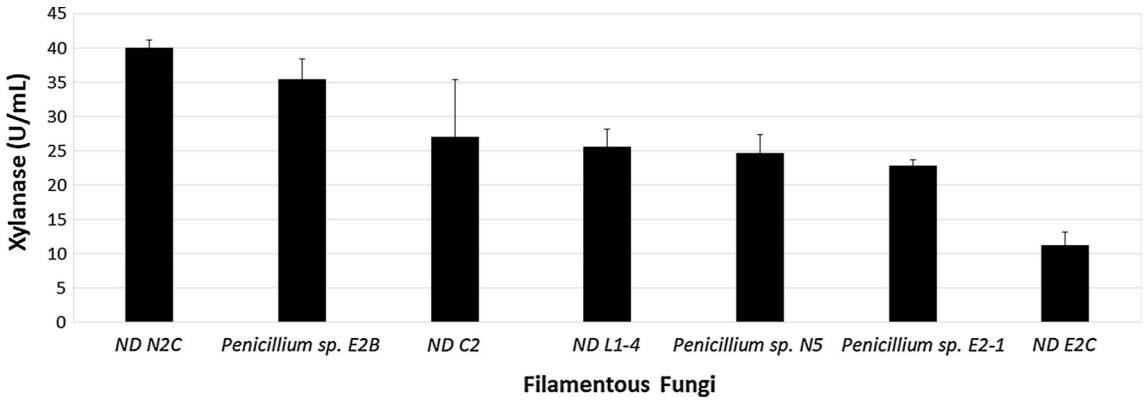

Fig. 3 Xylanase production by Antarctic filamentous fungi (values ≥ 10 U mL^{-1}) after 7 days of cultivation at 20.0 °C and 140 rpm

(*n* = 7), *Nacella* sp. (*n* = 2), ascidian (*n* = 2), lichens (*n* = 17), whale bones (*n* = 10), ornithogenic soil (*n* = 5), and wood (*n* = 4).

A total of 42 filamentous fungi able to produce L-ASNase in solid medium were identified using molecular taxonomy: *Geomyces* sp. (*n* = 11), *Pseudogymnoascus* sp. (*n* = 1), *Cosmospora* sp. (*n* = 7), *Hypocrea* sp. (*n* = 2), *Cadophora luteo-olivacea* (*n* = 3), *Cadophora malorum* (*n* = 6), *Cladosporium* sp. (*n* = 4), *Penicillium* sp. (*n* = 5), *Pseudeurotium* sp., *Oidiodendron* sp., and *Acremonium* sp. (*n* = 1).

Among the total of filamentous fungi that exhibited L-ASNase activity in liquid medium (*n* = 8), three were identified as *Cosmospora* sp. (all isolated from whale bones: OB4B, OB1B, and OB2), two as *Penicillium* sp. (recovered from sea star: C2 and E2B), and one as *Geomyces* sp. (recovered from *Salpa* sp.: S2B) (Fig. 4). The isolates OB7A (recovered from whale bones) and L2-16A (recovered from lichen) could not be identified using molecular taxonomy. *Penicillium* sp. C2 and the unidentified fungus OB7A showed the highest asparaginase activities (Fig. 4). *Penicillium* sp. isolates C2 and E2B were also able to produce xylanase.

Discussion

Taxonomic evaluation

All filamentous fungi identified in the present study belong to the phylum Ascomycota. Previous studies reported the prevalence of Ascomycota among the filamentous fungi found in the Antarctic environments (Arenz et al. 2006; Arenz and Blanchette 2009; Santiago et al. 2016), including studies related to next-generation DNA sequencing (Dreesens et al. 2014; Pudasaini et al. 2017). Filamentous fungi belonging to the phylum Basidiomycota are rarely found in Antarctica (Arenz and Blanchette 2009), the representatives of this phylum were mainly composed of yeasts (Ludley and Robinson 2008; Duarte et al. 2013, 2016). However, the predominance of ascomycetes observed in the present study

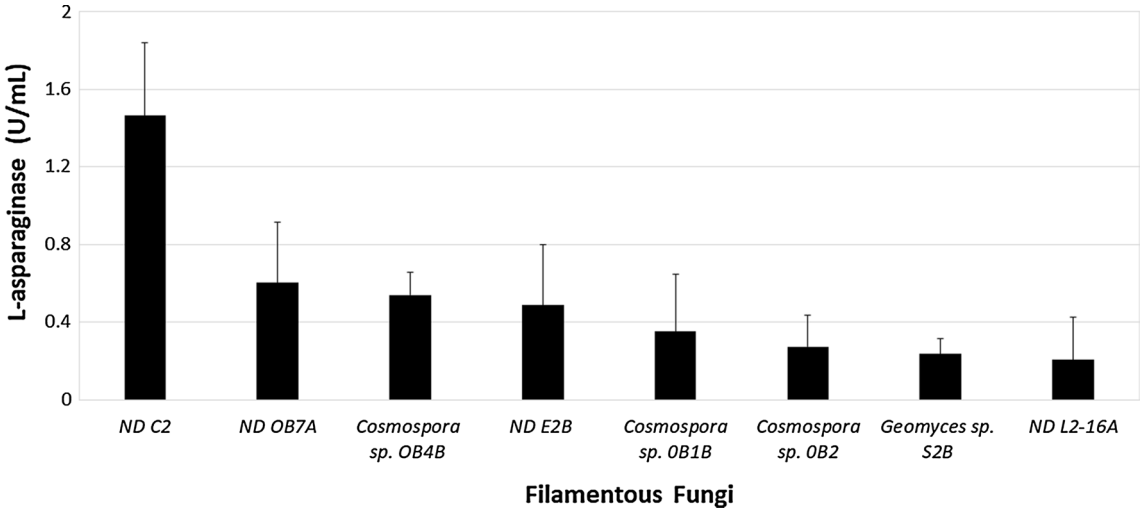

Fig. 4 L-ASNase produced by Antarctic filamentous fungi after 7 days of cultivation at 20.0 °C and 140 rpm

may be due to the culture medium used for fungal isolation, since representatives of ascomycetes show faster growth on PDA than basidiomycetes.

Based on sequencing and phylogenetic analyses, 12 different genera were identified, all of them previously reported in the Antarctic environments. The most abundant genus was *Cadophora*, followed by *Geomyces*, *Penicillium, Cosmospora,* and *Cladosporium.* With the exception of the genus *Cosmospora*, whose representatives were recovered only from terrestrial samples, all the other representatives of the most abundant genera were isolated from terrestrial and marine samples, suggesting they are widespread and adapted to the Antarctic extreme conditions.

According to Arenz et al. (2006), representatives of *Cadophora* have been reported as wood decomposers in the Antarctic environments, and in the present study, eight *Cadophora* isolates were recovered from wood samples. *Cadophora* is commonly found in Antarctic soils and has a broad growth temperature ranging from − 3.0 to 45.0 °C (Onofri et al. 2004). The predominance of this genus may suggest that it is an important microorganism in nutrient cycling in the polar regions.

The genus *Geomyces* presents psychrophilic features and halotolerance (Godinho et al. 2013). Species belonging to this genus have been reported by Zhdanova et al. (2000) to grow in high levels of radiation (greater than 220 mR h^{-1}, measured as the level of gamma radiation). These properties can explain their high abundance in Antarctic samples.

Many species in the genera *Penicillium* and *Cladosporium* are psychrotolerant, often found in different environments, including the Antarctic and Arctic polar regions (Mcrae et al. 1999; Held et al. 2006; Sonjak et al. 2006; Gonçalves et al. 2013, 2015). According to Onofri et al. (2004), the species *Cladosporium cladosporioides* can grow in temperatures ranging from 0.0 to 32.0 °C with optimum temperatures ranging from 20.0 to 28.0 °C. Additionally, representatives of the genus *Penicillium* have a strong capacity to form spores and to tolerate saline conditions (Menezes et al. 2010).

Despite this, in the present study, the genus *Cosmospora* were recovered only from terrestrial Antarctic samples. Representatives of this genus were reported from samples from Antarctic lakes (Gonçalves et al. 2012). Additionally, they were also found in the rhizosphere soil of *Deschampsia antarctica* (Gonçalves et al. 2015), one of the two vascular plants that inhabit the maritime Antarctic. The majority of *Cosmospora* species is the sexual stage of *Acremonium*-like or *Fusarium-like* fungi (Gräfenhan et al. 2011). Representatives of these genera are psychrotrophic and are reported also in Arctic soil (Ali et al. 2013).

Most fungi reported from Antarctica are considered asexual. This fact can be explained by a survival strategy, since asexual reproduction can be completed in a shorter time and without high metabolic costs. Some exceptions of sexual fungi have been reported, such as *Thelebolus*, frequently isolated from Arctic and Antarctic environments (Hoog et al. 2005; Ruisi et al. 2007), and *Cosmospora*.

Among all fungi identified in this study, a large amount came from terrestrial samples, the majority were isolated from wood and lichen. Antarctic lichens have been reported as a natural microhabitat with favorable conditions for microbial survival. Santiago et al. (2016) identified 74 taxa from 21 genera of filamentous fungi and yeasts recovered from Antarctic lichens, *Pseudogymnoascus*, *Thelebolus*, and *Antarctomyces* are the most abundant genera. In addition, Duarte et al. (2016) recovered 200 isolates of yeasts from Antarctic lichen samples collected

from different islands (South Shetland Archipelago), six of them are considered potential new species.

Many new fungal species recovered from Antarctic environments were recently described (Laich et al. 2013, 2014; Zhang et al. 2014; Turchetti et al. 2015; de Menezes et al. 2017), highlighting the relevance of the studies related to the diversity and exploitation of Antarctic-derived fungi. In the present study, the isolates *Pseudeurotium* sp. M14 and *Oidiodendron* sp. A11-M1 showed low similarity with sequences deposited in GenBank. In this sense, they may represent new species. However, further taxonomic studies should be done in order to confirm this hypothesis.

Cold-adapted enzymes

Considering the enzyme-producing ability of microorganisms, the properties of cold-adapted enzymes, and that fungi from maritime Antarctica are mainly classified as psychrotolerant, the selection approach applied in the present study was carried out at 15.0, 20.0, and 25.0 °C.

Different groups of fungi have been reported as producers of ligninolytic enzymes. Among them, the basidiomycetes (white rot fungi) are considered the most efficient in the breakdown of lignocellulosic materials (Bonugli-Santos et al. 2010a). In the present study, a higher number of fungi from the terrestrial Antarctic samples showed the ability to produce laccase and LiP at 15.0 and 25.0 °C. However, the number of marine-derived fungi able to produce these enzymes was also significant.

Despite the relevance of the ligninolytic enzymes for bioremediation of environmental pollutants and industrial applications (Bonugli-Santos et al. 2010b), studies related to the production of these enzymes by Antarctic fungi are scarce (Duarte et al. 2018). The ability of fungi from Antarctic origin to degrade environmental pollutants was reported by Gerginova et al. (2013) and Litova et al. (2014). Some of the genera identified in the present study (e.g., *Geomyces* and *Thelebolus*) were isolated from Antarctic soils contaminated with oil (Kerry 1990), highlighting their potential for oil tolerance/degradation. Additionally, the fungus *Geomyces* sp. M22 showed the ability to produce ligninolytic enzymes, which are one of the microbial pathways for pollutant degradation.

The largest number of fungi able to produce xylanase was recovered from marine sources. Bugni and Ireland (2004) highlighted the biotechnological potential of the marine microorganisms, based on their adaptation to the saline and pressure conditions that can lead to the production of natural products different from those produced by their terrestrial counterparts.

Xylanase have many industrial applications, including bread-making to help the dough rise (Courtin and Delcour 2002), pulp pre-bleaching in paper production (Bajpai 2004), as an animal feed additive to improve digestibility (Cowieson et al. 2006), production of ethanol (Polizeli et al. 2005), and in the synthesis of xylitol (Granström et al. 2007). However, there are few studies reported in the literature related to the production of this enzyme by Antarctic fungi (Duarte et al. 2018). According to Bradner et al. (1999) and Del-Cid et al. (2014), filamentous fungi from Antarctic ornithogenic soil and from marine sponges were reported as xylanase producers, including representatives of the genera *Alternaria, Cladosporium, Penicillium, Phoma,* and *Trichoderma*. In the study reported by Del-Cid et al. (2014), the fungus *Cladosporium* sp. recovered from Antarctic marine sponge was reported as a great producer of this enzyme at low temperatures when pure xylans from hardwoods (birchwood and beechwood) were used as carbon sources.

L-Asparaginase is a relevant medical enzyme, seeing that it is used as an anti-tumoral medicine in the treatment of lymphoblastic leukemia and lymphosarcoma. L-ASNase catalyzes the hydrolysis of L-asparagine into L-aspartic acid and ammonia. The depletion of L-asparagine in blood blocks the protein synthesis and leads to DNA and RNA synthesis inhibition, resulting in cellular apoptosis (Lopes et al. 2017). The high number of Antarctic filamentous fungi that were selected as L-ASNase producers (57%) in the first screening in solid medium, revealed the potential of these microorganisms. The microbial production of this enzyme is cost effective and eco-friendly, and the production of L-ASNase by an eukaryotic organism could avoid allergenic effects that are caused by L-ASNase industrially produced by prokaryotic organisms (Theantana et al. 2007).

Considering the potentially different properties of the microbial cold-adapted enzymes, the collection of Antarctic fungi structured from the marine and terrestrial samples can be considered relevant for biotechnological exploitation and is available for new studies. Additionally, fungal strains selected in the present study may produce enzymes with kinetic parameters important for specific biotechnological applications. In this sense, further studies related to low temperature activity, enzymatic optimization, and characterization should be performed.

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Compliance of ethical standards

Conflict of interest The authors declare no conflict of interest.

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