Antarctic Science 31(1), 3–12 (2019) © Antarctic Science Ltd 2018 doi:10.1017/S0954102018000421

Biodiversity and enzymes bioprospection of Antarctic filamentous fungi

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Abstract: Antarctica is one of the most suitable locations for the bioprospecting of psychrotrophic fungi, which play a key role in the nutrient cycle and organic material mineralization in cold environments. These actions mainly take place via the production of several cold-active extracellular enzymes. The aim of this study was to investigate the diversity of filamentous fungi from King George Island (25 De Mayo Island), Antarctica and their ability to produce extracellular hydrolytic enzymes at low temperatures. A total of 51 fungal isolates were obtained from 31 samples. Twelve genera were identified, with seven among the *Ascomycota* (*Cadophora*, *Helotiales*, *Monographella*, *Oidodendron*, *Penicillium*, *Phialocephala*, *Phialophora*, *Phoma* and *Pseudogymnoascus*), one *Basidiomycota* (*Irpex*) and two *Mucoromycota* (*Mortierella* and *Mucor*). *Monographella lycopodina* and *Mucor zonatus*, not previously reported in Antarctica, were identified. Nine isolates could not be identified to genus level and may represent novel species. Most of the studied fungi were psychrotrophic (76.5%). Nevertheless, only five isolates were able to grow at 35°C, 15°C being the optimal growth temperature for 65% of the fungal isolates. Results from enzyme production at low temperature revealed that the Antarctic environment contains metabolically diverse fungi, which represent potential tools for biotechnological applications in cold regions.

Received 13 February 2018, accepted 4 October 2018, first published online 19 December 2018

Key words: biotechnology, mycology, prospection, psychrophilic

Introduction

The Antarctic continent presents one of the harshest environments on this planet. It is cold and dry areas present a climate that it is not uniform, and, in that way, several main climatic zones can be recognized (Continental High Plateau, Continental Low Plateau, Continental High Latitude Coast, Continental Low Latitude Coast, Antarctic Peninsula, Antarctic Islands, Sub-Antarctic Islands). The existing environmental conditions in Antarctica - low temperature, water availability and precipitation; continuous freeze-thaw cycles; high UV incidence and intense winds - comprise a complex set of environmental conditions that make life difficult for animals as well as plants (Bokhorst *et al.* 2007). Life in Antarctica is dominated by microbes, as they show an adequate level of adaptation to the environment that helps them to prevail in the extreme conditions (Ruisi *et al.* 2007).

Microorganisms that colonize these environments are able to develop even at 0°C and can be classified as strict psychrophiles if their optimum growth temperature is 15°C or below, showing a maximal temperature for growth at about 20°C, or as psychrotolerants (psychrotrophics), which have the ability to grow at low temperatures, having optimal and maximal growth temperatures above 15°C and 20°C, respectively (Fernàndez *et al.* 2017). They play a key role in the nutrient cycle and organic material mineralization in cold environments such as the Arctic and Antarctica. These capabilities are mainly related to the production of several extracellular enzymes known as cold-adapted or cold-active enzymes. As explained by Gerday *et al.* (2000), these enzymes present a higher catalytic efficiency than those showing optimal temperatures between 25°C and 50°C (mesophiles) at temperatures lower than 20°C. Hence, these enzymes are a promising tool for industrial processes that demand elevated enzymatic activity at low temperature. Examples of these enzymes are: cellulase, amylase, inulinase, protease, isomerase and lipase, which have been used in the food, soap and biofuel industries (Margesin & Feller 2010).

For these reasons, the search and study of cold-adapted microorganisms has increased considerably in the last decade. Several Antarctic biotopes were investigated in the search of cold-adapted fungi (Ruisi *et al.* 2007). The major fungal phyla, *Ascomycota*, *Basidiomycota*, *Zygomycota*, *Chytridiomycota* and *Glomeromycota*, are well represented in the Antarctic continent (Godinho *et al.* 2013).

Based on this background, the objective of the present study was the isolation, identification and study of the exoenzyme production of psychrophilic/psychotropic fungi obtained from an Antarctic area located on King George Island. The study was focused on certain enzymatic activities in a collection of 51 isolates.

Materials and methods

Soil sampling and fungal isolation

Soil samples were collected during the 2013–2014 summer (December 2013–March 2014) in a radius of 5 km from the Argentinean Scientific Research Station, Carlini, on Potter Cove, King George Island (62°14'18"S, 58°40'00"W) (Fig. 1).

Samples were collected from several locations around the cove, including an ornithogenic site near the beach, a human refuge, two human-impacted areas (outside the station kitchen and near the fuel storage tanks), and a large and pristine vegetated area (Tres Hermanos Hill and nearby coastal soils) covered by *Deschampsia antarctica* Desv., lichens and mosses. Samples (*c.* 10 g) were aseptically taken from soil at 0–10 cm depth. After collection, the samples were stored in sealed sterile bags or sterile flasks and transported to the station, where they were kept at 4°C until processed for incubation and isolation.

Isolation was performed on solid media, using a diluted yeast morphology medium (DYM) (composition in g l^{-1}: yeast extract 0.03, malt extract 0.03, peptone 0.05, dextrose 0.1, agar 15; pH adjusted to 4.5 by addition of HCl 1 N (to allow the growth of fungi instead of bacteria)). The isolation protocol followed two parallel procedures. First, a portion of each sample was excised under aseptic conditions, using a sterile spoon or spatula, and directly spread onto Petri plates containing DYM. Simultaneously, another portion of the same sample was re-suspended in a minimal volume of saline solution supplemented with 1% Tween 20 and then homogenized in a vortex mixer for 15 min. After that, 100 µl of the resulting homogenate was spread onto Petri plates with DYM.

The plates were incubated at 15°C for 7–21 days under natural lighting conditions. Actively growing fungi colonies were taken from the plates and subcultured onto fresh potato dextrose agar (PDA) plates as individual isolates.

All fungal isolates were maintained on PDA medium at 4°C in the Microbiological Resources Center Culture Collection (MIRCEN) of the PROIMI-CONICET Institute and in the Culture Collection in the Argentinean Antarctic Institute (IAA).

Molecular identification of selected isolates

Genomic DNA extraction was performed using a commercial kit (FastDNA™ Spin Kit, MP Biomedicals).

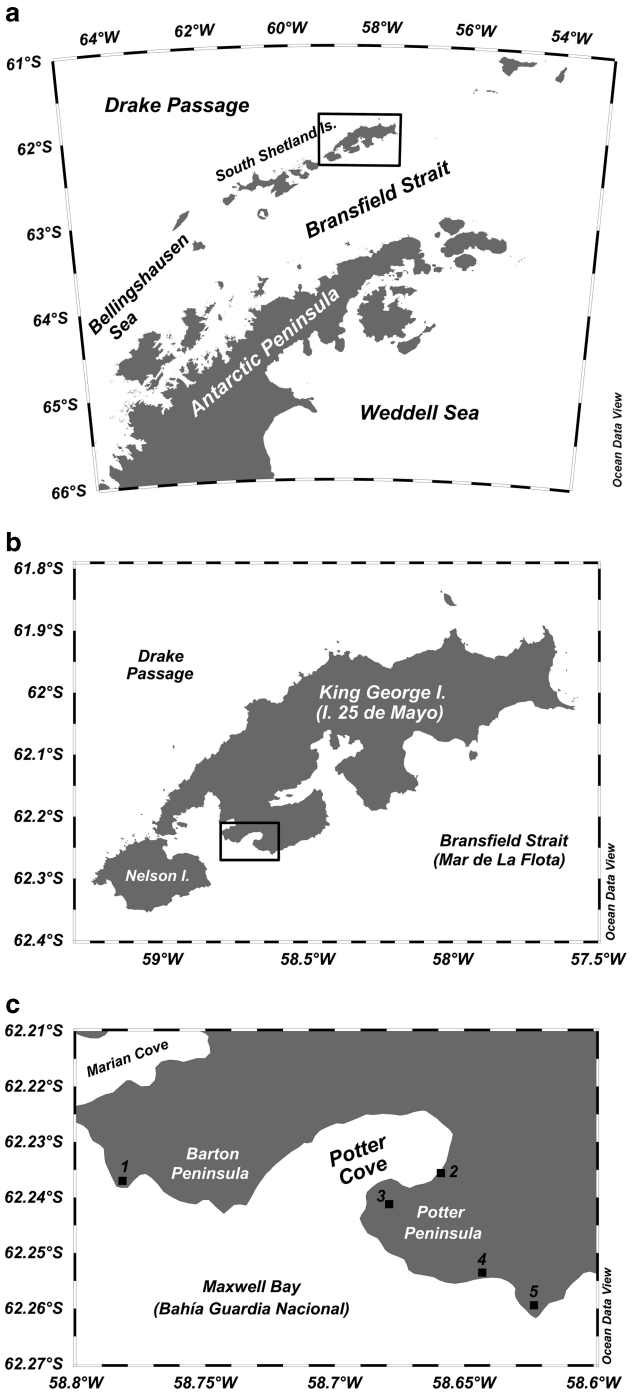

Fig. 1. The sampling area on King George Island, South Shetland Islands, Potter Cove (62°14'18"S, 58°40'00"W) with sites marked. Sampling sites: 1. Nesting penguins in Barton Peninsula (Antarctic Specially Protected Area (ASPA) 171), 2. Carlini Station facilities, 3. Tres Hermanos Hill, 4. Elephant Refuge (ASPA 132), 5. Stranger Point (ASPA 132).

The divergent domain at the 5' end of the LSU rDNA gene (around 600 base pairs) was symmetrically amplified with primers NL-1 (5'-GCATATCAATAAGCGGAGGAAAAG) and NL-4 (5'-GGTCCGTGTTTCAAGACGG)

https://doi.org/10.1017/S0954102018000421 Published online by Cambridge University Press

according to standard methods, as described by Kurtzman *et al.* (2011). This set of primers was chosen after trying to amplify the ITS region using ITS1 and ITS4 primers, which resulted in the production of several bands in some of the isolates (data not shown), as a consequence of unexpectedly high single nucleotide polymorphisms, a phenomenon previously reported by Simon & Weiß (2008). The PCR products were purified and sequenced in MACROGEN (Korea). Sequences were analysed and edited, when necessary, using DNA Dragon software (Hepperle 2011). DNA sequences were submitted to GenBank under accession numbers listed in Table I. Strain identifications were performed by comparison with the GenBank and UNITE databases. An identity criterion of ≥99% was employed to identify strains at the species level. Taxonomy was checked against Kurtzman *et al.* (2011). Sequences showing 97–98% identity were tentatively identified to the genus level. Sequences showing less than 97% identity were considered unidentified.

Screening of lytic enzymes production

Seven hydrolytic activities were tested in triplicate on fungal isolates growing on solid media at 15°C: amylase, cellulase, lipase, esterase, protease, laccase and xylanase. In all cases exoenzymatic activity was quantified as the diameter in mm of the halo (either colouration or decolouration) around the colony, from the edge of the colony to the edge of the halo.

For amylase, cellulase and xylanase activity, fungal isolates were grown in a YMD medium (1:10) with starch ($2\,g\,l^{-1}$), carboxymethylcellulose ($5\,g\,l^{-1}$), and xylane ($5\,g\,l^{-1}$), respectively, instead of glucose. To measure the activity after incubation, for amylase and xylanase, 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 (Carrasco *et al.* 2012). For cellulase, 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 (Carrasco *et al.* 2012).

For protease and lipase activity, fungal isolates were grown in YMD medium (1:10) supplemented with skimmed milk ($10\,g\,l^{-1}$) and olive oil (4% v/v), and rhodamine B 0.01%, respectively. Positive protease activity was defined as a clear halo around the colony on a white opaque background (Rovati *et al.* 2010) whereas positive lipase activity was indicated as fluorescent halos around the colonies under UV exposure (Gopinath *et al.* 2005).

For esterase activity, fungal isolates were grown in a medium composed of: bacto peptone, ($10\,g\,l^{-1}$); NaCl, ($5\,g\,l^{-1}$); $CaCl_2{\bullet}2H_2O$, ($4\,g\,l^{-1}$) and Tween 80, ($10\,g\,l^{-1}$). Esterase activity was evidenced as a white precipitate around the colony (Carrasco *et al.* 2012). Finally, for laccase activity, the fungal isolates were grown in medium composed of: glucose, ($2\,g\,l^{-1}$); bacto peptone ($0.1\,g\,l^{-1}$); yeast extract, ($0.01\,g\,l^{-1}$) and ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)) ($1\,g\,l^{-1}$). Laccase activity was indicated as green halos around the colonies after incubation (Maza *et al.* 2014).

Growth temperature range

The effect of temperature on the growth of the isolates was investigated on PDA plates. Fungi (pre-grown on PDA plates) were used to inoculate two replicates per strain at each temperature (given below) on 24 multi-well plates filled with 1 ml of PDA agar. Plates were incubated at 5°C, 15°C, 25°C and 35°C. Growth was monitored every seven days up to 21 days, to avoid missing any slow-growing fungi at a specific temperature. Growth was expressed as the fungal colony diameter in mm (to assess optimal growth temperature, colony diameter (at each temperature) was recorded after 7 days of incubation). To classify fungi as psychrophilic or psychrotrophic, the presence or absence of growth after 21 days was considered. For microorganisms able to grow at low temperature, two classifications were used: psychrophilic (any fungi not able to grow at or above 25°C) and psychrotrophic (any fungi able to grow at or above 25°C).

Results

A total of 31 samples from pristine and anthropized sites were used. It is worth mentioning that some samples were taken from soils impacted by oil spills over recent years.

After 7–21 days of incubation, 51 fungal morphotypes were recovered as pure cultures and deposited at the MIRCEN and IAA culture collections (Table I). When analysing the origins of the samples (Table II), it was noticed that most isolates came from substrates associated with high organic matter content: 34% of the isolates ($n = 18$) were found in samples associated with lichen, moss and *Deschampsia antarctica*; 28% ($n = 15$) came from samples such as soil or mud with penguin faeces, feathers, wood from shipwrecks, decaying seals' bodies; 28% ($n = 15$) came from soil or mud obtained near the station's kitchen exit or near the fuel tanks, and were consequently considered as anthropogenic-impacted. Finally, only 8% ($n = 4$) came from soils related to neither the station nor areas with high organic matter content.

The 51 isolates were ascribed to 12 different genera. Nine genera belonged to the phylum *Ascomycota* (*Cadophora*, *Helotiales*, *Monographella*, *Oidodendron*, *Penicillium*, *Phialocephala*, *Phialophora*, *Phoma* and *Pseudogymnoascus*), one genus to the phylum *Basidiomycota* (*Irpex*) and other two

https://doi.org/10.1017/S0954102018000421 Published online by Cambridge University Press

Table I. Molecular identification of the isolated fungi.

Isolate ID	Presumptive identification	*Genbank accession no.	Identification NCBI	% Identity	Identification UNITE	%Identity
226	*Cadophora malorum*	MF154657	*Mycochaetophora gentianae* strain: MAFF 239231	99	*Cadophora malorum*	100
232	*Cadophora malorum*	MF154659	*Mycochaetophora gentianae* strain: MAFF 239233	99	*Cadophora malorum*	100
319	*Helotiales* sp.	MF154662	*Coleophoma ericicola* strain CBS 301.72	94	Unidentified fungi	100
342	*Irpex* sp.	MF154672	*Dacryobolus montanus* isolate Yuan5673	98	*Irpex oreophilus*	98
14	*Monograpehlla lycopodina*	MF154669	*Monographella lycopodina* isolate LL	99	*Microdochium lycopodinum*	99
20	*Monograpehlla lycopodina*	MF154665	*Monographella lycopodina* isolate LL	99	*Microdochium lycopodinum*	99
145	*Mortierella* sp.	MF154623	*Mortierella parvispora* strain CBS 311.52	98	*Mortierella* sp.	98
348	*Mortierella* sp.	MF154652	*Gamsiella multidivaricata* strain CBS 227.78	93	*Mortierella* sp.	94
237	*Mucor hiemalis*	MF154643	*Mucor hiemalis f. hiemalis* strain CBS 201.65	99	*Mucor hiemalis*	99
23	*Mucor zonatus*	MF154622	*Mucor zonatus* strain CBS 148.69	99	*Mucor* sp.	99
302	*Oidodendron* sp.	MF154629	*Parafabraea eucalypti* culture-collection CBS:124810	94	*Oidiodendron truncatum*	98
142	*Penicilium* sp.	MF154654	*Penicillium clavigerum* strain NRRL 1003	99	*Penicillium* sp.	99
11	*Penicillium* sp.	MF154664	*Penicillium kojigenum* strain NRRL 3442	99	*Penicillium lanosum*	99
21	*Penicillium* sp.	MF154632	*Penicillium kojigenum* strain NRRL 3442	99	*Penicillium lanosum*	99
331	*Penicillium* sp.	MF154637	*Penicillium clavigerum* strain NRRL 1003	99	*Penicillium* sp.	100
132	*Phialocephala* sp	MF154658	*Mycochaetophora gentianae*	99	Phialocephala sp.	100
178	*Phialocephala* sp.	MF154624	*Mycochaetophora gentianae* strain: MAFF 239231	98	*Phialocephala* sp.	100
225	*Phialocephala* sp.	MF154630	*Mycochaetophora gentianae* strain: MAFF 239231	99	*Phialocephal*a sp.	100
230	*Phialocephala* sp.	MF154670	*Mycochaetophora gentianae* strain: MAFF 239231	99	*Phialocephala* sp.	100
343	*Phialocephala* sp.	MF154650	*Mycochaetophora gentianae* strain: MAFF 239231	99	*Phialocephala* sp.	100
179	*Phialophora* sp.	MF154639	*Mycochaetophora gentianae* strain: MAFF 239231	99	*Phialophora* sp.	99
229	*Phialophora* sp.	MF154645	*Mycochaetophora gentianae* strain: MAFF 239231	99	*Phialophora* sp.	99
332	*Phialophora* sp.	MF154648	*Mycochaetophora gentianae* strain: MAFF 239231	99	*Phialophora* sp.	100
327	*Phoma* sp.	MF154627	*Peyronellaea prosopidis* strain CPC 21698	99	*Phoma* sp.	99
111	*Pseudogymnoascus pannorum*	MF154641	*Pseudogymnoascus destructans* 20631-21	98	*Pseudogymnoascus pannorum*	99
234	*Pseudogymnoascus pannorum*	MF154621	*Pseudogymnoascus destructans* 20631-21	99	*Pseudogymnoascus pannorum*	99
241	*Pseudogymnoascus pannorum*	MF154646	*Pseudogymnoascus destructans* 20631-21	99	*Pseudogymnoascus pannorum*	100
242	*Pseudogymnoascus pannorum*	MF154655	*Pseudogymnoascus destructans* 20631-21	99	*Pseudogymnoascus pannorum*	99
243	*Pseudogymnoascus pannorum*	MF154656	*Pseudogymnoascus destructans* 20631-21	99	*Pseudogymnoascus pannorum*	99
258	*Pseudogymnoascus pannorum*	MF154631	*Pseudogymnoascus destructans* 20631-21	99	*Pseudogymnoascus pannorum*	99
277	Pseudogymnoascus pannorum	MF154661	*Pseudogymnoascus destructans* 20631-21	99	*Pseudogymnoascus pannorum*	99
294	*Pseudogymnoascus pannorum*	MF154626	*Pseudogymnoascus destructans* 20631-21	99	*Pseudogymnoascus pannorum*	99
295	*Pseudogymnoascus pannorum*	MF154628	*Pseudogymnoascus destructans* 20631-21	99	*Pseudogymnoascus pannorum*	99
301	*Pseudogymnoascus pannorum*	MF154634	*Pseudogymnoascus destructans* 20631-21	99	*Pseudogymnoascus pannorum*	99
306	*Pseudogymnoascus pannorum*	MF154651	*Pseudogymnoascus destructans* 20631-21	99	*Pseudogymnoascus pannorum*	99
311	*Pseudogymnoascus pannorum*	MF154640	*Pseudogymnoascus destructans* 20631-21	99	*Pseudogymnoascus pannorum*	100
315	*Pseudogymnoascus pannorum*	MF154660	*Pseudogymnoascus destructans* 20631-21	99	*Pseudogymnoascus pannorum*	100
320	*Pseudogymnoascus pannorum*	MF154671	*Pseudogymnoascus destructans* 20631-21	99	*Pseudogymnoascus pannorum*	100
322	*Pseudogymnoascus pannorum*	MF154636	*Pseudogymnoascus destructans* 20631-21	99	*Pseudogymnoascus pannorum*	100

325	*Pseudogymnoascus pannorum*	MF154647	*Pseudogymnoascus destructans* 20631-21	99	*Pseudogymnoascus pannorum*	100
326	*Pseudogymnoascus pannorum*	MF154667	*Pseudogymnoascus destructans* 20631-21	99	*Pseudogymnoascus pannorum*	100
347	*Pseudogymnoascus pannorum*	MF154644	*Pseudogymnoascus destructans* 20631-21	99	*Pseudogymnoascus pannorum*	100
353	*Pseudogymnoascus pannorum*	MF154668	*Pseudogymnoascus destructans* 20631-21	99	*Pseudogymnoascus pannorum*	100
55	Unidentified fungi	MF154666	*Phialocephala virens*	95	Unidentified fungi	100
184	Unidentified fungi	MF154625	Coleophoma ericicola strain CBS 301.72	95	Unidentified fungi	100
204	Unidentified fungi	MF154653	*Phialocephala virens* strain: CBS 452.92	95	Unidentified fungi	100
212	Unidentified fungi	MF154638	*Arbusculina fragmentans* strain CCM F-13486	96	Unidentified fungi	100
227	Unidentified fungi	MF154635	*Fusarium larvarum var. rubrum* strain F-267,622	97	Unidentified fungi	100
313	Unidentified fungi	MF154642	*Mycochaetophora gentianae* strain: MAFF 239231	97	Unidentified fungi	99
335	Unidentified fungi	MF154633	*Fusarium larvarum var. rubrum* strain F-267,622	93	Unidentified fungi	95
351	Unidentified fungi	MF154663	*Arbusculina fragmentans* strain CCM F-13486	96	Unidentified fungi	100

*Presumptive identification corresponds to the database identification with higher percentage of identity and coverage (data not shown).

genera to the phylum *Mucoromycota* (*Mortierella* and *Mucor*). Nine isolates could not be identified to genus level (Table I).

Only 25 isolates were identified to species level, and they belonged to five species *Pseudogymnoascus pannorum* (Link) Minnis & D.L. Lindner, *Cadophora malorum* (Kidd & Beaumont) W. Gams, *Monographella lycopodina* Jaklitsch, Siepe & Voglmayr, *Mucor zonatus* Milko and *Mucor hiemalis* Wehmer. The other isolates were only identified to genus level. More studies and other genes sequences are needed to reach the species level in those cases.

Pseudogymnoascus pannorum was the fungus most frequently found in this study, with 19 isolates. *Monographella lycopodina* ($n = 2$), *Mucor zonatus* ($n = 1$) and *Mucor hiemalis* ($n = 1$) came from the same sample, a mix of *Deschampsia antarctica* and lichen. Two isolates of *Cadophora malorum* came from impacted areas (around the kitchen exit and fuel tanks).

Results from the enzymatic screening are presented in Table II. Xylanase activity was the most abundant enzymatic activity, produced by 90% ($n = 46$) of the isolates. Cellulase activity was evidenced by 80% ($n = 41$) of the isolates whereas 78% of the isolates ($n = 40$) were proteolytic. Amylase was produced by 76% of the isolates ($n = 39$), Lipase by 71% ($n = 36$) and esterase, another lipolytic enzyme by only 53% ($n = 27$). Finally, laccase, a lignocellulolytic enzyme typical of basidiomycete fungi, was detected only in 37% of the isolates ($n = 19$).

Temperature response showed 23.5% ($n = 12$) were classified as psychrophiles and 76.5% ($n = 39$) were classified as psychrotrophic. Among the last group, only five isolates were able to grow at 35°C. The optimum temperature for growth was 15°C for 65% ($n = 33$) of the isolates and 25°C for 31% ($n = 16$). Only one isolate (*Mortierella* sp. CAV1-348) grew better at 5°C and isolates *Mucor zonatus* CAV1-23 and *Mucor hiemalis* CAV1-237 presented the same growth in the range 5–25°C.

Discussion

King George Island is the biggest of the South Shetland Islands, north-west of the Antarctic Peninsula, has a mild macroclimate that is strongly buffered by the surrounding ocean (Krishnan *et al*. 2011). Its climate is typical of the peri-Antarctic islands: wet, windy and cold. It has an average temperature of 1–3°C in summer and -7°C in winter (Kostadinova *et al*. 2009). Soil temperature in winter ranges between -5°C and -9°C buffered from lower air temperatures by the snow layer (Krishnan *et al*. 2011). In summer, maximal soil temperatures range from 14–26°C (Martínez-Álvarez *et al*. 2017). While these habitats clearly experience chronically low mean temperatures, various other aspects of temperature stress, including long-term (seasonal or annual) and short-term (daily or weekly) variation, also present challenges to soil microbiota.

https://doi.org/10.1017/S0954102018000421 Published online by Cambridge University Press

Table II. Enzyme activity of the fungal isolates.

Identification	Sample Site	Organic content	Classification**	Growth temperature* ° C				Extracellular Enzymatic Production***						
				5	15	25	35	amylase	cellulase	lipase	esterase	protease	xylanase	laccase
Cadophora malorum CAV1-226	soil near fuel tanks (human impacted area)	High	Psychrotrophic	3	5	6	1	3	3	1	1	1	3	-
Cadophora malorum CAV1-232	soil near kitchen exit (human impacted area)	High	Psychrotrophic	4	8	12	-	3	3	1	1	1	3	-
Helotiales sp. 1-319	soil near seal corps in decomposition	High	Psychrophilic	1	2	-	-	2	-	3	1	1	3	1
Irpex sp. CAV1-342	soil near seal corps in decomposition	High	Psychrotrophic	-	-	2	-	-	-	3	-	1	3	-
Monograpehlla lycopodina CAV1-14	lichen and Deschampsia antarctica	High	Psychrophilic	-	16	-	-	1	1	-	1	-	-	1
Monograpehlla lycopodina CAV1-20	lichen and Deschampsia antarctica	High	Psychrophilic	5	6	-	-	-	3	-	-	1	3	1
Mortierella sp. CAV1-145	moss	High	Psychrophilic	-	16	-	-	-	1	2	-	-	-	-
Mortierella sp. CAV1-348	soil near seal corps in decomposition	High	Psychrophilic	6	-	-	-	-	1	2	-	-	-	-
Mucor hiemalis CAV1-237	lichen and Deschampsia antarctica	High	Psychrotrophic	16	16	16	5	-	1	1	1	-	1	-
Mucor zonatus CAV1-23	lichen and Deschampsia antarctica	High	Psychrotrophic	16	16	16	7	-	1	1	1	-	1	-
Oidiodendron sp. CAV1-302	lichen and moss	High	Psychrotrophic	3	6	2	-	-	-	-	1	1	-	-
Penicilium sp. CAV1-142	moss	High	Psychrotrophic	7	8	8	-	3	1	2	-	1	3	-
Penicillium sp. CAV1-11	lichen and Deschampsia antarctica	High	Psychrotrophic	4	8	-	-	1	3	1	-	1	3	-
Penicillium sp. CAV1-21	lichen and Deschampsia antarctica	High	Psychrophilic	5	9	-	-	3	3	2	-	1	3	-
Penicillium sp. CAV1-331	soil far from the station (non-impacted area)	Low	Psychrotrophic	6	8	9	-	3	-	1	-	-	3	1
Phialocephala sp. CAV1-132	soil near the station (human impacted area)	High	Psychrotrophic	3	5	11	-	3	2	-	-	-	3	1
Phialocephala sp. CAV1-178	soil near fuel tanks (human impacted area)	High	Psychrotrophic	3	7	12	-	3	2	2	1	1	3	2
Phialocephala sp. CAV1-225	soil near fuel tanks (human impacted area)	High	Psychrotrophic	5	8	16	1	3	2	-	1	1	3	3
Phialocephala sp. CAV1-230	soil near the station (human impacted area)	High	Psychrotrophic	3	6	8	1	3	3	2	1	1	3	-
Phialocephala sp. CAV1-343	soil near seal corps in decomposition	High	Psychrotrophic	1	4	8	-	3	3	2	-	1	3	1
Phialophora sp. 1-332	soil far from the station (non-impacted area)	Low	Psychrotrophic	4	6	11	-	3	-	2	-	1	3	-
Phialophora sp. CAV1-179	mud near the station (impacted area)	High	Psychrotrophic	4	6	10	-	3	-	2	-	1	3	-
Phialophora sp. CAV1-229	soil near kitchen exit (impacted area)	High	Psychrotrophic	2	4	9	-	-	-	1	-	-	2	-
Phoma sp. CAV1-327	soil near nesting birds	High	Psychrotrophic	10	12	16	-	3	3	-	-	-	3	1
Pseudogymnoascus pannorum CAV1-111	soil near fuel tanks (human impacted area)	High	Psychrotrophic	4	5	-	-	3	1	1	1	1	3	1
Pseudogymnoascus pannorum CAV1-234	soil far from the station (non-impacted area)	Low	Psychrotrophic	3	7	2	-	1	1	-	1	1	1	-
Pseudogymnoascus pannorum CAV1-241	lichen and Deschampsia antarctica	High	Psychrotrophic	5	6	2	-	3	-	-	1	1	3	2
Pseudogymnoascus pannorum CAV1-242	lichen and Deschampsia antarctica	High	Psychrotrophic	5	6	1	-	1	1	-	1	1	1	-
Pseudogymnoascus pannorum CAV1-243	lichen and Deschampsia antarctica	High	Psychrotrophic	5	8	-	-	3	2	1	1	1	3	2
Pseudogymnoascus pannorum CAV1-258	mud near nesting birds	High	Psychrotrophic	6	9	2	-	3	2	1	1	1	3	-
Pseudogymnoascus pannorum CAV1-277	soil far from the station (non-impacted area)	Low	Psychrotrophic	3	5	-	-	3	3	1	1	1	3	-
Pseudogymnoascus pannorum CAV1-294	soil and feathers near nesting birds	High	Psychrophilic	5	6	-	-	3	1	3	1	1	3	-
Pseudogymnoascus pannorum CAV1-295	soil near shipwreck (human impacted area)	High	Psychrotrophic	3	7	1	-	2	3	1	1	1	3	2
Pseudogymnoascus pannorum CAV1-301	lichen and moss	High	Psychrotrophic	-	4	-	-	2	3	1	-	1	3	-
Pseudogymnoascus pannorum CAV1-306	lichen and moss	High	Psychrophilic	3	5	-	-	1	1	-	1	1	1	-

Pseudogymnoascus pannorum CAV1-311	soil and feathers near nesting birds	High	Psychrotrophic	2	5	-	-	1	1	-	1	1	1	-
Pseudogymnoascus pannorum CAV1-315	soil near shipwreck (human impacted área)	High	Psychrophilic	1	4	-	-	2	3	1	1	1	3	3
Pseudogymnoascus pannorum CAV1-320	soil near seal corps in decomposition	High	Psychrotrophic	4	8	2	-	3	3	1	1	1	3	1
Pseudogymnoascus pannorum CAV1-322	lichen	High	Psychrotrophic	2	9	-	-	1	1	-	-	1	1	1
Pseudogymnoascus pannorum CAV1-325	penguin feces	High	Psychrotrophic	4	5	-	-	3	3	1	1	1	3	**2**
Pseudogymnoascus pannorum CAV1-326	lichen near nesting birds	High	Psychrophilic	2	6	-	-	3	3	1	1	1	3	-
Pseudogymnoascus pannorum CAV1-347	soil near shipwreck (human impacted área)	High	Psychrotrophic	5	7	1	-	3	3	1	1	1	3	-
Pseudogymnoascus pannorum CAV1-353	soil near the station (human impacted area)	High	Psychrophilic	4	8	-	-	-	-	1	-	1	-	-
Unidentified fungi CAV1-55	lichen and Deschampsia antarctica.	High	Psychrophilic	2	7	-	-	3	1	1	-	1	3	-
Unidentified fungi CAV1-184	lichen and Deschampsia antarctica	High	Psychrotrophic	-	4	-	-	-	2	2	-	-	2	-
Unidentified fungi CAV1-204	soil near fuel tanks (human impacted area)	High	Psychrotrophic	-	2	-	-	2	-	-	-	1	2	-
Unidentified fungi CAV1-212	mud near the station (human impacted area)	High	Psychrotrophic	6	8	9	-	-	1	1	-	1	1	-
Unidentified fungi CAV1-227	soil near kitchen exit (human impacted area)	High	Psychrotrophic	3	5	7	-	-	3	1	1	1	3	-
Unidentified fungi CAV1-313	soil and feathers near nesting birds	High	Psychrotrophic	7	10	-	-	3	2	-	-	1	3	1
Unidentified fungi CAV1-335	soil near shipwreck (human impacted area)	High	Psychrotrophic	4	4	6	-	2	3	1	-	1	3	-
Unidentified fungi CAV1-351	soil near the station (human impacted area)	High	Psychrotrophic	-	3	-	-	3	2	-	-	-	3	1

*The diameter of the fungi colony at each temperature at 7 days of incubation was measured in mm and related to the optimal growth temperature. **The classification was made based on the growth at 21 days of incubation. ***Exoenzymatic activities were quantified as the radius (in mm) of the halo (of either colouration or decolouration) around the colony as explained in material and methods.

Results presented here helped to identify fungi not previously reported on the continent, proving the value of culture-based studies to determine the ecology of filamentous fungi on the island. The presence of several research stations in this environmentally sensitive area represents a source of high environmental impact. The effect of the human impact areas as well as those with natural high organic content (nesting birds, soil covered with *Deschampsia antarctica*, lichen or moss) on the mycodiversity was reflected by the fact that 93% of the fungi (37% from human-impacted areas and 56% from naturally high organic soils) were isolated from these areas. This observation is in accordance with Ding *et al.* (2016), who reported that cultivable fungal distribution and abundance patterns in Antarctic soils is positively correlated with soil nutrients, content and moisture. As a rule, differences in the composition of Antarctic soils lead to distinctive and characteristic microbial distributions.

In this work, the genus *Pseudogymnoascus* (formerly *Geomyces*) was the most frequently isolated and abundant taxon, with 19 members of *Pseudogymnoascus pannorum* isolated. The genus *Pseudogymnoascus* is one of the most reported fungal genera in Antarctica (Gonçalves *et al.* 2012, Ding *et al.* 2016) comprising psychrotrophic/psychrophilic fungi common in cold environments, and ubiquitous to soils not only from Antarctica, but also Arctic and Alpine soils (Hayes *et al.* 2012). In Antarctica, this genus has been isolated from different habitats (Ding *et al.* 2016). *Pseudogymnoascus* species were reported by Arenz *et al.* (2006) to have the ability to colonize and utilize several carbon sources, and therefore play and important role in the decomposition and nutrient cycle in Antarctica. From our 19 isolates of *Pseudogymnoascus pannorum*, six could produce all the enzymes tested in this work. Fungi with a broad enzymatic competence possess high eco-nutritional versatility: the ability to survive in a vegetative state, and to overcome environmental changes that might normally be harmful, increasing their survival chances under unfavourable environmental conditions (Cooke & Rayner 1984).

Like *Pseudogymnoascus*, members of the genus *Penicillium* have frequently been isolated from Antarctic soils (Onofri *et al.* 2007). This genus is ubiquitous, with a worldwide distribution, but the frequency of occurrence and range of the individual species may be more limited (McRae *et al.* 1999). Moreover, it is hard to determine if particular species are actually endemic to Antarctica due to their extensive distribution across the planet and their cold tolerance. Penicillia are easily dispersed and several Antarctic isolates could certainly represent the result of involuntary human contamination of natural Antarctic environments being transported with human foods, clothes, vehicles and other man-made materials (Comerio & Mac Cormack 2004). In this sense, for the *Penicillium* genus (with the ascomycetous state *Eupenicillium*), only one novel species

https://doi.org/10.1017/S0954102018000421 Published online by Cambridge University Press

has been described from cold regions. However, although isolated from Antarctica, this species, *Penicillium antarcticum*, is not particularly psychrotolerant (McRae *et al.* 1999). So, no penicillia have been reported as geographically confined to the Arctic or Antarctica, and even *Penicillium antarcticum* occurs worldwide. Despite this, Gunde-Cimerman *et al.* (2003) indicate that there may be several novel cold-tolerant species of *Penicillium* in Antarctic and Arctic areas. Based on the growth temperature experiments (Table II), one isolate was psychrophilic (*Penicillium* sp. CAV1-21) and the other three (*Penicillium* sp. CAV1-11, CAV1-142 and CAV1-331) were psychrotrophic.

The class *Leutuomycetes* is represented by *Phialocephala* a common plant-root endophyte that is widespread in sub-Antarctic ecosystems and is also present in continental Antarctica (Newsham *et al.* 2009). In this work, five isolates ascribed to these genera, all from soil samples, exhibited most of the evaluated enzymatic activities. Two of the isolates (*Phialocephala* sp. CAV1-225 and CAV1-230) were able to grow, although poorly, at 35°C.

Amongst the *Mucorales*, *Mortierellaceae* and *Mucoraceae* are the most frequently recorded families in Antarctica (Ruisi *et al.* 2007). Genera belonging to these families are known to produce many mitotic spores that have resistance-reproductive structures that enhance survival opportunities (Onofri *et al.* 2004). They exhibit worldwide distribution and are regarded as psychrotolerant organisms (Onofri *et al.* 2004). In this work, members of two genera (*Mortierella* and *Mucor*) belonging to these families were isolated. *Mortierella* has frequently been obtained from different habitats from Antarctica (Ding *et al.* 2016). In the present study, members of the genus were isolated from moss and soil with an elevated organic content (*Mortierella* sp. CAV1-145 and CAV1-348). These isolates were classified as psychrophilic and showed two of the seven evaluated enzyme activities, cellulase and lipase. The other *Mucoromycota* species isolated were *Mucor hiemalis* CAV1-237 and *Mucor zonatus* CAV1-23, which both came from a sample containing moss and from a *Deschampsia antarctica* specimen. These isolates showed psychrotrophic behaviour and grew particularly well at 35°C. Fungi belonging to this genus were previously isolated in Antarctica (Gesheva & Negoita 2012). Nevertheless, this work is the first report of *Mucor zonatus* in the Antarctic continent.

The size of halos and colonies were used to compare enzymatic activities and colony growth under different conditions. Several investigators have described the sensitivity of this method for semi-quantitative determinations and it has been used successfully for identification of novel activities from environmental isolates of microorganisms (Li *et al.* 2009). Two isolates, which presented six of the seven evaluated enzymes, and characterized as psycrothrophic, were identified as *Cadophora malorum* (CAV1-226 and CAV1-232). Four other isolates (CAV1-332, CAV1-179, CAV1-229 and CAV1-337) were identified to genus level as *Phialophora* sp. (synonym for *Cadophora* sp.). These isolates were also classified as psychrotrophic. Fungi belonging to genus *Cadophora* were previously reported on a range of different substrates (Arenz *et al.* 2006). The presence of previously reported species of *Cadophora* in Antarctica and the prevalence of these fungi at many locations in the continent suggests that they are indigenous (Blanchette *et al.* 2004).

Other genera reported in Antarctica were also identified in this work, as *Phoma* sp. CAV1-327, isolated from soil, and *Oidiodendron* sp. CAV1-302, isolated from lichens and mosses. Both isolates were classified as psychrotrophic. These genera were previously isolated from different substrates such as wood from shipwrecks or structures (Held & Blanchette 2017), soil (Kochkina *et al.* 2014) moss (Hirose *et al.* 2016) and macroalgae (Godinho *et al.* 2013). In the case of isolate *Irpex* sp. CAV1-342, this genus was previously identified in Antarctic soil from the volcano Mount Erebus (Connell & Staudigel 2013).

Two of the isolates (CAV1-14 and CAV1-20) identified as *Monographella lycopodina*, represent the first time this genus has been isolated in Antarctica. *Monographella* taxa include important plant pathogens, particularly of grasses and cereals. In cold to temperate regions *Monographella nivalis*, also known as 'pink snow mould', is an economically important disease of wheat and barley (Jewell & Hsiang 2013). In this work, they were isolated from cellulolytic substrates as *Deschampsia antarctica* and lichen, and, as expected, presented two enzymatic activities related to mycorrhiza, laccase and cellulase.

Most of the isolates were successfully classified at either the species or genus level based on the LSU sequences of standard isolates deposited in the GenBank and the UNITE database according to the BLAST results, except for the isolates CAV1-55, CAV1-184, CAV1-204, CAV1-212, CAV1-227, CAV1-313, CAV1-335, CAV1-351 and CAV1-113. Also, isolate CAV1-319 showed 99% identity with the *Helotiales* sp. I12F-02289 sequence isolated from bryophytes as culturable endophytic fungi in Antarctica (Zhang *et al.* 2013) and was further identified at order level as *Helotiales* sp. These fungi might represent novel species.

The results obtained from enzyme production suggest that several of these fungi could be used for industrial purposes, for example in large-scale microbial fermentation for the production of cold-active enzymes. In recent years, the use of lipases and proteases in the soup and dairy industries has become a reality. Other lytic enzymes as amylase, pectinase and cellulase have also proven to be helpful in making juice products more palatable and of better appearance (Pandey *et al.* 2000). These are only a few examples of the usefulness of some of the enzymes evaluated in this work.

The results presented here suggest that most of the fungal isolates from maritime Antarctica were psychrotrophs, in accordance with those reported in related studies (Ruisi *et al.* 2007). The predominance of psychrotrophic fungi in

https://doi.org/10.1017/S0954102018000421 Published online by Cambridge University Press

surface soils from cold environments has been previously noted and it is attributable to seasonal and diurnal increases in soil temperature due to solar radiation that favours the dominance of the eurythermal cold-adapted microorganisms over the stenothermal ones represented by the strict psychrophiles (Robinson 2001). This ability also suggests an interesting way to select cold-adapted microorganisms for biotechnological purposes, because psychrotrophic microorganisms can grow at low temperatures but do not have the limited growth at moderate temperatures that the strict psychrophiles have.

Conclusion

This study investigated the taxonomic diversity and enzyme production in solid media of 51 cultivable fungi isolated from samples taken on King George Island. In agreement with previous research, some characteristic fungal taxa found in this region belong to cold-adapted and cosmopolitan taxa. Furthermore, some other taxa were found that have not been previously observed in Antarctica (*Monographella lycopodina* and *Mucor zonatus*) as well as several not fully identified fungal isolates, which will be more thoroughly investigated. The isolation of mainly psychrothrophic fungi having at least one cold-active enzymatic activity raises their potential for use in biotechnological/industrial processes at low and moderate temperatures.

Acknowledgments

This work was supported by Instituto Antártico Argentino/ Dirección Nacional del Antártico (IAA/DNA), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT, Project PICTO 2010-0124), Universidad de Buenos Aires (UBA, Project 20020130100569BA) and Universidad Nacional de Tucumán (UNT). The authors would like to thank the reviewers for their time spent on reviewing this manuscript and their helpful comments towards improving the article.

Authors contribution statement

All authors conceived and planned the experiments. Martorell and Fernández carried out the experiments. Ruberto, Figueroa and Mac Cormack. contributed to the interpretation of the results. Martorell took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

Details of data deposit

DNA sequences were submitted to GenBank under Accession Numbers listed in Table I.

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