Extremophiles
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Phenol degradation and heavy metal tolerance of Antarctic yeasts

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Abstract In cold environments, biodegradation of organic pollutants and heavy metal bio-conversion requires the activity of cold-adapted or cold-tolerant microorganisms. In this work, the ability to utilize phenol, methanol and n-hexadecane as C source, the tolerance to different heavy metals and growth from 5 to 30 °C were evaluated in cold-adapted yeasts isolated from Antarctica. Fifty-nine percent of the yeasts were classified as psychrotolerant as they could grow in all the range of temperature tested, while the other 41% were classified as psychrophilic as they only grew below 25 °C. In the assimilation tests, 32, 78, and 13% of the yeasts could utilize phenol, n-hexadecane, and methanol as C source, respectively, but only 6% could assimilate the three C sources evaluated. In relation to heavy metals ions, 55, 68, and 80% were tolerant to 1 mM of Cr(VI), Cd(II), and Cu(II), respectively. Approximately a half of the isolates tolerated all of them. Most of the selected yeasts belong to genera previously reported as common for Antarctic soils, but several other genera were also isolated, which contribute to the knowledge of this cold environment mycodiversity. The tolerance to heavy metals of the phenol-degrading cold-adapted yeasts illustrated that the strains could be valuable as inoculant for cold wastewater treatment in extremely cold environments.

Keywords Phenol · Heavy metals · Bioremediation · Tolerance · Yeasts · Antarctica

Introduction

A large fraction of our planet (>80%) exhibit temperature values permanently below 5 °C, including areas, such as deep oceans, glaciers, and Polar Regions (Margesin et al. 2007). Microorganisms that colonize these environments can develop even at 0 °C and are classified as strict psychrophiles if its optimum growth temperature is 15 °C or below and a maximal temperature for growth at about 20 °C, or as psychrotolerants (psychrotrophics) which have the ability to grow at low temperatures, but have optimal and maximal growth temperatures above 15 and 20 °C, respectively (Morita 1975; Moyer and Morita 2007; Robinson 2001; Hassan et al. 2016). These microorganisms that have adapted their cellular processes to thrive at temperatures near freezing point of water (D'Amico et al. 2006) assume an essential contribution to nutrient recycling and mineralization of organic matter in ecosystems with extreme cold weather. These metabolic capabilities are performed through a special class of enzymes generally called "cold enzymes", as these molecules have a higher catalytic efficiency at temperatures below 20 °C and can show unusual substrate specificities (Gerday et al. 2000).

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Bacteria are the most studied extremophiles microorganisms, whereas fungi and yeasts have been in a minor proportion (Margesin and Miteva 2011). Members of the Fungi kingdom represent a very diverse group and considering their presence in extreme environments is one of the main examples of microorganisms that have biotechnological potential not yet studied.

In the last years, it has been suggested that psychrophilic yeasts may be better adapted to low temperatures than bacteria (Shivaji and Prasad 2009); for this reason, the number of reports describing the isolation of yeasts from cold environments is increasing (Connel et al. 2008; Shivaji and Prasad 2009; Margesin and Feller 2010; Thomas-Hall et al. 2010; Carrasco et al. 2012; Rovati et al. 2013, Zalar and Gunde 2014; Turchetti et al. 2008). The bulk of these reports is focused on their biotechnological potential and industrial uses (Buzzini et al. 2012; Hamid et al. 2014).

Antarctica is one of the most suitable sites for the search and study of psychrophilic microorganisms. It is permanently subjected to temperatures that rarely exceed the freezing point of water; however, its geographic location, its difficult access, and the international diplomatic and political treatment of their land and seas make it a world's region still very little explored in terms of microbial biodiversity.

A moderate level of anthropogenic contamination has been reported in Antarctica due to global warming, population growth in the stations and industrial activity in far countries of both the Southern and Northern hemispheres (Curtosi et al. 2007; Bargagli 2008; Corsolini 2009; Lo Giudice et al. 2013). Toxic compounds, such as heavy metals, antibiotics, pesticides, and other persistent pollutants, can be transferred to the antarctic continent through natural processes by mass flows in the atmosphere and oceans. Although strict guidelines are provided in the Protocol of Environmental Protection to the Antarctic Treaty for protection of the antarctic environment (ATCM 1991), several contamination events are produced by improper disposal practices and/or incineration of wastes at the research stations (De Souza et al. 2006; Corsolini 2009; Lo Giudice et al. 2013).

Phenols and its derivatives are troublesome environmental pollutant commonly found in many industrial effluents. Despite being toxic, phenol can be utilized by microbes as carbon and energy sources (Gibson et al. 1968). Only members of a few yeast genera (*Rhodotorula, Trichosporon*, and *Candida*) were reported as capable to metabolize phenolic compounds as a sole carbon and energy source (Santos and Linardi 2001; Alexieva 2002; Chen 2002).

The aerobic biodegradation at low temperatures of many components of petroleum hydrocarbons, including n-alkanes, aromatic, and polycyclic aromatic hydrocarbons (PAHs), has been reported in Arctic, Alpine, and Antarctic environments (Margesin and Schinner 1998). A wide variety of bacteria, fungi, and algae can metabolize aliphatic and aromatic hydrocarbons (Alexander 1999). Filamentous fungi are known for their potential to degrade PAHs (Gramss et al. 1999). There is, however, little information about the hydrocarbon-degradative potential of yeasts. Because the exposure to heavy metals represents a stress condition, the greater capacity of some cold-adapted isolates towards metal tolerance may be attributed to their ability to survive under extreme temperatures, since the expression of some genes could be involved in mechanisms battling against both stress factors (Abe and Minegishi 2008; Singh et al. 2012).

The objective of this work was to isolate and identify cold-adapted yeasts from different biotopes of sub-Antarctic zones, to evaluate their assimilation of some organic pollutants (phenol, methanol, and n-hexadecane) as carbon source, and to investigate their tolerance towards heavy metal ions at low temperatures.

Materials and methods

Soil sampling and fungal isolation

Soil samples were collected during the 2013–2014 austral summer (December 2013–March 2014) near the Argentinean Scientific Research Station, Carlini, located on the Potter Cove, 25 de Mayo/King George Island (62°14′18″S, 58°40′00″W) (Fig. 1).

Samples were collected from a range of locations around the cove, including ornithogenic soils near the beach (close to nesting birds areas in Punta Stranger and Burton Beach), a human refuge (Refugio Elefante) near a large penguin colony, two human-impacted areas (under the main dining room and near the diesel fuel storage tanks), and a largely pristine and naturally vegetated area (Tres Hermanos Hill and nearby beaches).

Samples (around 10 g) were taken from soil at a depth of 0–10 cm, using a sterile spatula. After collected, the samples were stored in sealed sterile bags or sterile flasks and immediately transported to the station, where they were kept at 4 °C until processed for incubation and isolation.

For yeasts isolation, samples were subjected to two parallel procedures. A portion of each soil sample was excised under aseptic conditions, using a sterile spoon or spatula, and directly spread onto Petri plates containing Yeast Morfology Medium (YM) diluted 1:10 (composition in g L^{-1}: yeast extract 0.3, malt extract 0.3, peptone 0.3, dextrose 0.5, agar 15, pH adjusted to 4.5).

Simultaneously, another portion of the same sample was resuspended in a minimal volume of saline solution supplemented with 1% tween 20 and then homogenized in a

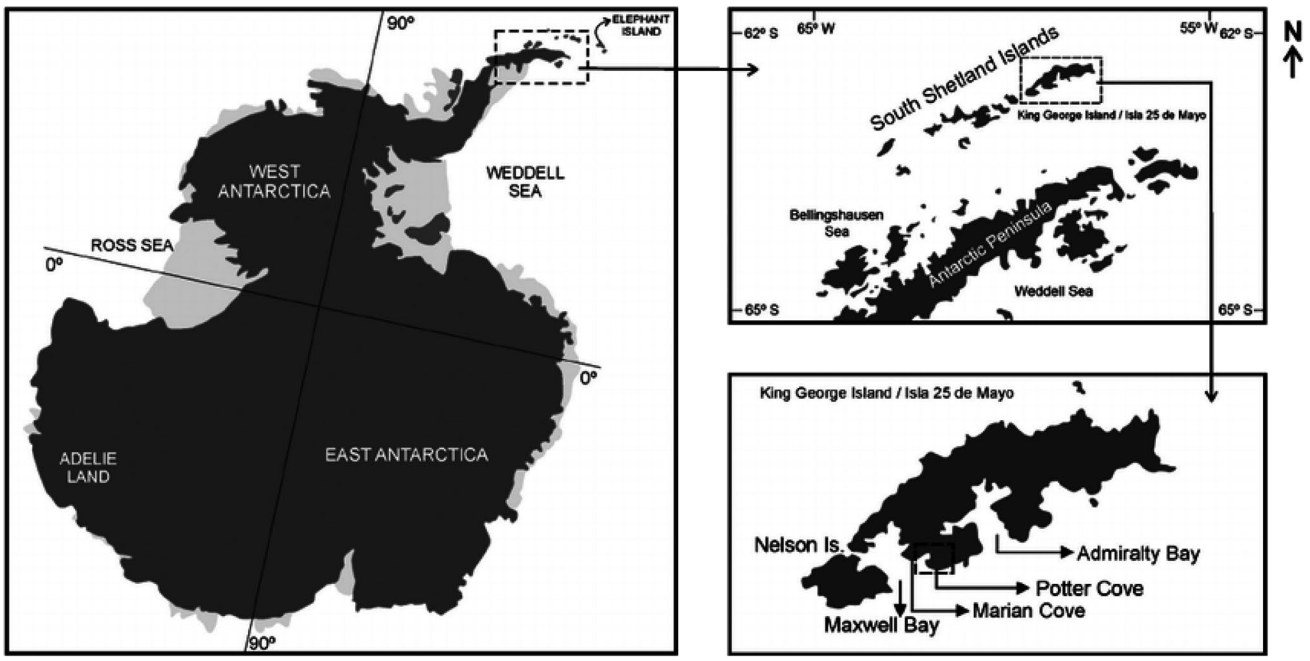

Fig. 1 Studied area in King George Island/Isla 25 de Mayo, South Shetland Islands, with indication of the sampling site, Potter Peninsula (62°14′18″S, 58°40′00″W) (Lana et al. 2014)

vortex mixer for 15 min. After that, 100 µL of the resulting homogenate was spread onto Petri plates with YM 1:10. In all cases, the media pH were adjusted to 4.5 to facilitate the growth of yeasts instead of bacteria. The plates were incubated at 15 °C for 7–14 days in a room near the laboratory under natural lighting conditions (day and night cycles). Actively growing colonies were taken from the plates and subcultured onto fresh YM 1:10 agar plates as individual isolates.

Yeast isolates were deposited in the Microbiological Resources Center Culture Collection (MIRCEN) of PROIMI-CONICET Institute and in the Culture Collection in the Argentinean Antarctic Institute (IAA).

All yeast strains were maintained on isolation medium agar plates (without antibiotics) at 4 °C and transferred monthly.

Biochemical tests

To classify the isolates as basidiomycetaceous or ascomycetaceous, production of urease and Diazonium Blue B test was performed. For urease production, isolated were inoculated on Christensen agar medium (g L^{-1}: urea 20, peptone 1, NaCl 5, KH_2PO_4 2, agar 20, and phenol red were added in a concentration of 0.16 µg L^{-1}) and the appearance of a pink color in the agar was considered as a positive reaction. Diazonium Blue B (DBB) test was performed according to Hagler and Ahearn (1981). In these experiments, cultures were incubated at 15 °C.

Pollutants assimilation as carbon source

Biomass from 72 h cultures in YM broth was recovered by centrifugation and resuspended in sterile distilled water to be used as inoculant in the assimilation tests. YNB (Yeast Nitrogen Base, DIFCO) liquid medium containing $(NH_4)_2SO_4$ (0.6 g L^{-1}) was supplemented with either phenol (2.5 mM), methanol (50 mM), or n-hexadecane (1 g L^{-1}). These media were inoculated with 200 µL of yeast suspension ($DO_{600nm} = 0.8$) and incubated at 250 rpm and 15 °C. Media without carbon source were also prepared and used as control cultures. After 7 and 14 days, DO of the cultures was measured at $\lambda = 600$ nm. Cultures presenting growth values exceeding those exhibited by control cultures in 50% or more were considered as positives.

Heavy metal ions tolerance

Divalent copper and cadmium [Cu(II) and Cd(II)] and hexavalent chromium [Cr(VI)] tolerance was separately evaluated in agarized YM medium added with 1 mM (final concentration) of each metal ion. Isolates were inoculated, incubated at 15 °C, and checked for growth up to 14 days. Plates without metals ions were also inoculated as controls (Fernández et al. 2013).

Growth temperature range

The effect of temperature on the growth of the strains was investigated on agar plates. Loopful of microbial cells (pregrown on YM agar) was used to inoculate two replicates per strain and temperature on YM agar. Plates were incubated at 5, 15, 25, and 30 °C. Growth was monitored up to an incubation time of 7–21 days.

Molecular Identification of selected isolates

Genomic DNA extraction was performed according to Libkind et al. (2003). The divergent domain at the 5′ end of the LSU rDNA gene (around 600 bp) was symmetrically amplified with primers NL-1 (5′-GCATATCAA TAAGCGGAGGAAAAG) and NL-4 (5′-GGTCCGTGT TTCAAGACGG) according to the standard methods, as described by Kurtzman (2011). Sequences were analyzed, and edited if necessary using the DNA Dragon software. DNA sequences from all isolates were submitted to GenBank under Accession Numbers listed in Table 2. Strains identification was performed by comparison with the GenBank (only type strains) and AFToL databases. Arbitrarily, $a \geq 99\%$ identity criterion was employed to identify strains at the species level. Taxonomy was checked against Kurtzman (2011). Sequences showing 97–98% identity were tentatively identified to the genus level. Sequences showing less than 97% identity were considered unidentified.

Results and discussion

Sampling and isolation of strains

A total of 31 samples, including both, pristine and anthropized sites, were collected from different areas of 25 de Mayo/King George Island and processed, as described in "Materials and methods". Some samples corresponded to soils that suffered recent oil spills, which happened through the last years.

After 7–14 days of incubation, isolates were grouped based on their colony characteristics, such as pigmentation, shape, texture, elevation, size, and time of appearance. After this characterization scheme, 128 yeast morphotypes were recovered as pure cultures and deposited at the MIRCEN and IAA culture collections (Table 1). Based on the color of the colonies and the urease and DBB tests, 74% of the yeasts were classified as basidiomycetous. In concordance with Rovati et al. (2013), we hypothesized that the isolation medium employed, which has low carbon content, could have biased the results towards oligotrophic, slow-growing, metabolically diverse yeasts, and characteristics exhibited mainly by basidiomycetous genera.

Assimilation of pollutants as carbon source

Results on assimilation of phenol, methanol, and n-hexadecane as carbon source are shown in Table 1 for all the isolates. Methanol was the less frequently used as carbon sources by yeasts, and only 13% ($n=17$) of the isolates could assimilate it. Phenol and n-hexadecane were assimilated by 32 ($n=41$) and 78% ($n=100$) of the isolates, respectively. Only a group of eight isolates (approximately 6%) comprising both, asco and basidiomycetous genera, could assimilate the three evaluated carbon sources (see Tables 1, 2).

In this study, we paid special attention to those isolates able to assimilate phenol and phenolic compounds, as they are common constituents of wastewater from the oil industry. Due to their toxicity to microorganisms, phenolic compounds can cause the breakdown of wastewater-treatment plants by inhibition of microbial growth, even at concentration as low as 2 mM (Li and Humphrey 1989). For this reason, the isolated phenol-degrading microorganisms represents a valuable tool as potential cold-tolerant components of the phenol-containing wastewaters treatment plants (Viswanath et al. 2014).

Interestingly, in this study, we were able to isolate hydrocarbon-degrading yeasts not only from hydrocarbon-contaminated environments but also from pristine areas, which indicates the ubiquity of these cold-adapted hydrocarbon degrading. Other authors have reported isolation of microorganisms able to efficiently degrade crude oil hydrocarbons (Margesin and Schinner 1998) and phenol (Bastos et al. 2000) from uncontaminated environments. However, Aislabie et al. (2001) working with Antarctic soils from Scott Base and Marble Point detected culturable yeasts only in oil-contaminated soils but no in pristine control soils. These authors attributed the significant enhancement in numbers of culturable yeasts and filamentous fungi in oil-contaminated cold soils to the important role of fungi in the degradation of hydrocarbons or their metabolites.

Tolerance to heavy metals

Heavy metal tolerance screening on solid media was performed on Petri dishes at the final concentration of 1 mM of each metal ion [Cr(VI), Cd(II), Cu(II)]. Of all isolates studied, 55 ($n=70$), 68 ($n=87$), and 80% ($n=103$) were tolerant to Cr(VI), Cd(II), and Cu(II), respectively but half of the yeasts tolerate all of them. In addition, 20% ($n=25$) could be classified as sensitive, showing no growth after 14 days in any of the metals under study. Results have been depicted in the Table 1.

Soil as well as water contaminated by heavy metals leads to accumulation of these harmful ions in living beings through the food chain, which causes a negative

Table 1 Colony description, classification as asco- or basidiomycetous, assimilation of phenol, methanol and n-hexadecane as carbon source, tolerance of heavy metal, and growth temperature of yeasts isolates from 25 de Mayo Island

Number	Colony color*	Urease test**	DBB test***	Classification	Carbon source assimilation			Growth temperature					Metal tolerance		
					Methanol	Phenol	n-Hexadecane	5 °C	15 °C	25 °C	30 °C	Classification	$K_2Cr_2O_7$	$CdCl_2$	$CuSO_4$
1	White	Negative	–	Ascomycetous	Negative	Negative	Positive	–	+++	+	++	Psychrotolerant	–	–	–
2	Pink/orange	–	–	Basidiomycetous	Negative	Negative	Positive	+++	+++	+	+	Psychrotolerant	+	+++	+++
6	**Pink/orange**	–	–	**Basidiomycetous**	**Positive**	**Positive**	**Positive**	+	+++	–	–	**Psychrophilic**	-	+	++
8	Pink/orange	–	–	Basidiomycetous	Negative	Negative	Positive	+++	+++	++	+	Psychrotolerant	+	++	++++
9	**Pink/orange**	–	–	**Basidiomycetous**	**Negative**	**Positive**	**Positive**	++	+++	-	-	**Psychrophilic**	-	+++	+++
12	**White**	**Variable**	**Positive**	**Basidiomycetous**	**Negative**	**Positive**	**Negative**	+++	+++	+	+	**Psychrotolerant**	+	++	+++
13	**White**	**Variable**	**Negative**	**Ascomycetous**	**Negative**	**Positive**	**Negative**	+++	+++	+++	+++	**Psychrotolerant**	++	++	+++
15	White	Positive	Positive	Basidiomycetous	Negative	Negative	Negative	+++	+++	–	–	Psychrophilic	+	+	++
16	Pink/orange	–	–	Basidiomycetous	Negative	Negative	Positive	++	+++	+++	+++	Psychrotolerant	+	++	+++
17	White	Positive	Positive	Basidiomycetous	Negative	Negative	Negative	+++	+++	–	–	Psychrophilic	++	+	++
25	White	Positive	Negative	Ascomycetous	Negative	Negative	Positive	+++	+++	–	–	Psychrophilic	+	+	+
27	White	Positive	Negative	Ascomycetous	Negative	Negative	Positive	+++	+++	+	+++	Psychrotolerant	++	+++	+++
28	White	Positive	Positive	Basidiomycetous	NEGATIVE	Negative	Positive	+++	+++	++	+	Psychrotolerant	+	+++	+++
31	White	Positive	Positive	Basidiomycetous	Negative	Negative	Positive	+++	+++	+	–	Psychrophilic	+	+++	+++
37	**White**	**Positive**	**Positive**	**Basidiomycetous**	**Positive**	**Positive**	**Negative**	+++	+++	+	+	**Psychrotolerant**	++	+++	+++
44	White	Positive	Positive	Basidiomycetous	Negative	Negative	Positive	+++	+++	–	–	Psychrophilic	++	+++	+++
47	White	Positive	Positive	Basidiomycetous	Negative	Negative	Positive	++	+++	+++	+++	Psychrotolerant	++	++	+++
51	White	Positive	Positive	Basidiomycetous	Negative	Negative	Positive	+++	+++	–	–	Psychrophilic	–	–	–
53	**White**	**Positive**	**Positive**	**Basidiomycetous**	**Negative**	**Positive**	**Negative**	+++	+++	++	+	**Psychrotolerant**	+	++	+++
54	**White**	**Positive**	**Positive**	**Basidiomycetous**	**Positive**	**Positive**	**Positive**	+	+++	++	+++	**Psychrotolerant**	–	++	++
56	Pink/orange	–	–	Basidiomycetous	Negative	Negative	Positive	+	+++	–	+	Psychrophilic	–	–	–
59	**White**	**Variable**	**Negative**	**Ascomycetous**	**Positive**	**Positive**	**Positive**	+++	+++	+++	+++	**Psychrotolerant**	++	++	+++
60	Pink/orange	–	–	Basidiomycetous	Negative	Negative	Positive	+++	+++	+++	+	Psychrotolerant	+	++	+++
65	White	Positive	Positive	Basidiomycetous	Negative	Negative	Positive	–	+++	+	+	Psychrotolerant	–	–	–
67	White	Positive	Positive	Basidiomycetous	Negative	Positive	Negative	+++	+++	–	–	Psychrophilic	–	–	–
70	White	Positive	Positive	Basidiomycetous	Negative	Negative	Negative	+++	+++	+++	+++	Psychrotolerant	++	++	+++
82	White	Positive	Positive	Basidiomycetous	Negative	Negative	Positive	+++	+++	–	–	Psychrophilic	–	–	+
84	**White**	**Positive**	**Positive**	**Basidiomycetous**	**Negative**	**Positive**	**Positive**	+++	+++	++	+++	**Psychrotolerant**	++	++	+++
88	White	Variable	Positive	Basidiomycetous	Negative	Negative	Negative	+++	+++	++	++	Psychrotolerant	+	+++	+++
91	White	Negative	–	Ascomycetous	Negative	Negative	Positive	–	+++	+++	+++	Psychrotolerant	+	–	+
92	White	Positive	Positive	Basidiomycetous	Negative	Negative	Positive	++	+++	+	+	Psychrophilic	–	++	+++
94	White	Positive	Positive	Basidiomycetous	Negative	Positive	Negative	–	+++	+	–	Psychrophilic	–	–	–
97	White	Positive	Positive	Basidiomycetous	Negative	Negative	Positive	+++	+++	++	–	Psychrophilic	+	++	+++
98	White	Negative	–	Ascomycetous	Negative	Negative	Negative	++	+++	–	–	Psychrophilic	–	–	++

Table 1 (continued)

Number	Colony color*	Urease test**	DBB test***	Classification	Carbon source assimilation			Growth temperature					Metal tolerance		
					Methanol	Phenol	n-Hexadecane	5 °C	15 °C	25 °C	30 °C	Classification	$K_2Cr_2O_7$	$CdCl_2$	$CuSO_4$
99	**White**	**Negative**	–	**Ascomycetous**	**Negative**	**Positive**	**Positive**	++	+++	–	–	**Psychrophilic**	–	–	++
100	White	Positive	Negative	Ascomycetous	Negative	Negative	Positive	+++	+++	++	+	Psychrotolerant	+	++	+++
103	Pink/orange	–	–	Basidiomycetous	Negative	Negative	Positive	+	+++	+		Psychrophilic	–	–	–
105	Pink/orange	–	–	Basidiomycetous	Negative	Negative	Negative	+++	+++	+	–	Psychrophilic	–	–	–
107	Pink/orange	–	–	Basidiomycetous	Negative	Negative	Positive	+	+++	–	+	Psychrophilic	–	–	–
128	Pink/orange	–	–	Basidiomycetous	Negative	Negative	Positive	++	+++	+	+	Psychrophilic	–	++	++
130	Pink/orange	–	–	Basidiomycetous	Positive	Negative	Negative	+++	+++	–	–	Psychrophilic	–	++	+++
131	Pink/orange	–	–	Basidiomycetous	Negative	Negative	Positive	+++	+++	–	–	Psychrophilic	–	–	–
134	**White**	**Positive**	**Positive**	**Basidiomycetous**	**Negative**	**Positive**	**Positive**	+++	+++	+	+	**Psychrophilic**	–	+	+++
153	White	Positive	Positive	Basidiomycetous	Negative	Negative	Positive	+++	+++	+	+	Psychrophilic	+	++	+++
155	Pink/orange	–	–	Basidiomycetous	Negative	Negative	Positive	++	+++	–	–	Psychrophilic	–	++	++
157	White	Positive	Positive	Basidiomycetous	Negative	Negative	Positive	–	+++	+++	+	Psychrotolerant	–	–	–
159	White	Positive	Positive	Basidiomycetous	Negative	Negative	Negative	–	+++	++	++	Psychrotolerant	–	–	–
161	White	Positive	Positive	Basidiomycetous	Negative	Negative	Positive	+++	+++	+++	+++	Psychrotolerant	++	++	+++
162	White	Positive	Positive	Basidiomycetous	Negative	Negative	Positive	++	+++	–	+	Psychrophilic	+	++	+++
163	White	Positive	Positive	Basidiomycetous	Negative	Negative	Positive	+	+++	+	–	Psychrophilic	–	–	–
164	White	Negative	–	Ascomycetous	Negative	Negative	Negative	+++	+++	–	–	Psychrophilic	–	–	–
165	White	Positive	Positive	Basidiomycetous	Positive	Positive	Positive	+	+++	++	–	Psychrophilic	–	–	–
166	**White**	**Positive**	**Positive**	**Basidiomycetous**	**Negative**	**Positive**	**Positive**	+	+++	+	–	**Psychrophilic**	–	–	++
167	White	Positive	Positive	Basidiomycetous	Negative	Positive	Positive	+++	+++	–	–	Psychrophilic	–	–	–
168	Pink/orange	–	–	Basidiomycetous	Negative	Negative	positive	+++	+++	++	+++	Psychrotolerant	++	++	+++
171	**White**	**Negative**	**Negative**	**Ascomycetous**	**Positive**	**Positive**	**Negative**	+++	+++	+++	+++	**Psychrotolerant**	+++	++	++
172	**White**	**Positive**	**Positive**	**Basidiomycetous**	**Negative**	**Positive**	**Positive**	+++	+++	+++	+++	**Psychrotolerant**	+	++	+++
174	**White**	**Positive**	**Positive**	**Basidiomycetous**	**Negative**	**Positive**	**Positive**	+++	+++	+++	+++	**Psychrotolerant**	+++	++	++
176	White	Positive	Negative	Ascomycetous	Negative	Negative	Positive	+++	+++	+++	–	Psychrophilic	+	+++	+++
180	White	Positive	Positive	Basidiomycetous	Positive	Negative	Positive	–	+++	+	+	Psychrotolerant	–	–	–
182	White	Positive	Positive	Basidiomycetous	Negative	Negative	Positive	+++	+++	+++	–	Psychrophilic	–	++	+++
183	White	Positive	Positive	Basidiomycetous	Negative	Negative	Positive	+++	+++	–	–	Psychrophilic	–	–	–
185	Pink/orange	–	–	Basidiomycetous	Negative	Negative	Positive	++	+++	++	–	Psychrophilic	–	+	+++
186	White	Positive	Positive	Basidiomycetous	Negative	Negative	Positive	–	+++	–	++	Psychrotolerant	–	+	++
190	**White**	**Positive**	**Positive**	**Basidiomycetous**	**Negative**	**Positive**	**Positive**	+++	+++	+	+++	**Psychrotolerant**	+++	+++	+++
192	White	Positive	Negative	Ascomycetous	Negative	Negative	Negative	+++	+++	+	++	Psychrotolerant	+	++	+++
194	White	Positive	Positive	Basidiomycetous	Negative	Positive	Positive	–	+++	+	++	Psychrotolerant	–	–	–
197	**White**	**Positive**	**Positive**	**Basidiomycetous**	**Negative**	**Positive**	**Positive**	++	+++	+	++	**Psychrotolerant**	+	++	++
199	White	Positive	Positive	Basidiomycetous	Negative	Negative	Positive	++	+++	–	++	Psychrotolerant	+	+++	+++
200	White	Positive	Positive	Basidiomycetous	Positive	Positive	Negative	+++	+++	–	–	Psychrophilic	–	–	–

Table 1 (continued)

Number	Colony color*	Urease test**	DBB test***	Classification	Carbon source assimilation			Growth temperature					Metal tolerance		
					Methanol	Phenol	n-Hexadecane	5 °C	15 °C	25 °C	30 °C	Classification	$K_2Cr_2O_7$	$CdCl_2$	$CuSO_4$
201	White	Positive	Positive	Basidiomycetous	Negative	Negative	Positive	+++	+++	−	−	Psychrophilic	−	−	−
210	**White**	**positive**	**Negative**	**Ascomycetous**	**Negative**	**Positive**	**Positive**	+++	+++	+++	+++	**Psychrotolerant**	+	−	+++
211	**White**	**Positive**	**Positive**	**Basidiomycetous**	**Negative**	**Positive**	**Positive**	++	+++	++	+++	**Psychrotolerant**	++	−	++
214	White	Positive	Negative	Ascomycetous	Negative	Negative	Negative	+++	+++	++	+	Psychrotolerant	+	+++	+++
215	White	Negative	–	Ascomycetous	Negative	Negative	Negative	+++	+++	++	++	Psychrotolerant	+	++	+++
217	**Pink/orange**	–	–	**Basidiomycetous**	**Negative**	**Positive**	**Negative**	++	+++	+	++	**Psychrotolerant**	++	++	+++
235	**White**	**Negative**	**−–**	**Ascomycetous**	**Positive**	**Positive**	**Positive**	+++	+++	++	+++	**Psychrotolerant**	+++	++	+++
236	Pink/orange	–	–	Basidiomycetous	Negative	Negative	Positive	+++	+++	+++	++	Psychrotolerant	++	+	+
240	White	Positive	Negative	Ascomycetous	Negative	Negative	Positive	+	+++	+++	+++	Psychrotolerant	−	−	+++
243	**Pink/orange**	–	–	**Basidiomycetous**	**Negative**	**Positive**	**Positive**	+	+++	+	+++	**Psychrotolerant**	−	++	++
245	White	Negative	–	Ascomycetous	Negative	Negative	Negative	++	+++	+	+	Psychrotolerant	−	+	++
248	**Pink/orange**	–	–	**Basidiomycetous**	**Positive**	**Positive**	**Positive**	++	+++	++	++	**Psychrotolerant**	+	++	++
249	**White**	**Negative**	–	**Ascomycetous**	**Negative**	**Positive**	**Positive**	+++	+++	++	+	**Psychrotolerant**	++	+++	+++
250	White	Negative	–	Ascomycetous	Negative	Negative	Positive	++	+++	−	+++	Psychrotolerant	−	++	+++
251	**Pink/orange**	–	–	**Basidiomycetous**	**Negative**	**Positive**	**Positive**	+	+++	−	+	**Psychrotolerant**	−	++	++
252	**White**	**Negative**		**Ascomycetous**	**Positive**	**Positive**	**Positive**	++	+++	+++	+++	**Psychrotolerant**	+	+	++
257	**White**	**Positive**	**Positive**	**Basidiomycetous**	**Negative**	**Positive**	**Positive**	+++	+++	+++	+++	**Psychrotolerant**	++	++	+++
258	White	Positive	Positive	Basidiomycetous	Positive	Negative	Positive	+++	+++	+++	+	Psychrotolerant	−	++	++
259	White	Positive	Positive	Basidiomycetous	Negative	Negative	Positive	+++	+++	+	−	Psychrophilic	+	++	+++
260	White	Positive	Positive	Basidiomycetous	Negative	Negative	Positive	+++	+++	−	−	Psychrophilic	+	++	+++
261	White	Positive	Positive	Basidiomycetous	Negative	Negative	Positive	+++	+++	−	−	Psychrophilic	−	−	+++
263	White	Positive	Positive	Basidiomycetous	Negative	Negative	Positive	+++	+++	−	+	Psychrophilic	−	++	+++
264	**White**	**Positive**	**Positive**	**Basidiomycetous**	**Negative**	**Positive**	**Positive**	+++	+++	−	−	**Psychrophilic**	++	++	+++
265	**White**	**Positive**	**Positive**	**Basidiomycetous**	**Positive**	**Positive**	**Negative**	+++	+++	−	+	**Psychrophilic**	+	+++	+++
273	**White**	**Positive**	–	**Basidiomycetous**	**Negative**	**Positive**	**Positive**	+++	+++	+++	−	**Psychrophilic**	+	++	++
274	White	Positive	Positive	Basidiomycetous	Positive	Negative	Negative	−	+++	+++	++	Psychrotolerant	−	−	++
275	White	Positive	Positive	Basidiomycetous	Negative	Negative	Positive	+++	+++	++	−	Psychrophilic	−	++	++
276	**White**	**Positive**	**Positive**	**Basidiomycetous**	**Negative**	**Positive**	**Positive**	++	+++	+++	+++	**Psychrotolerant**	+++	+++	+++
278	Pink/orange	–	–	Basidiomycetous	Negative	Negative	Negative	++	+++	++	−	Psychrophilic	+	+++	+++
279	**Pink/orange**	–	–	**Basidiomycetous**	**Negative**	**Positive**	**Positive**	+++	+++	+++	++	**Psychrotolerant**	+	+++	+++
281	White	Negative	–	Ascomycetous	Negative	Negative	Positive	+++	+++	++	+	Psychrotolerant	−	−	−
284	White	Negative	–	Ascomycetous	Negative	Negative	Negative	+++	+++	++	+	Psychrotolerant	+	++	+++
288	White	Positive	–	Basidiomycetous	Negative	Negative	Negative	+++	+++	−	−	Psychrophilic	−	−	−
291	Pink/orange	–	–	Basidiomycetous	Negative	Negative	Positive	++	+++	+++	+	Psychrotolerant	−	++	+++
292	White	Variable	Negative	Ascomycetous	Negative	Negative	Positive	+++	+++	++	++	Psychrotolerant	+	++	+++
293	**Pink/orange**	–	–	**Basidiomycetous**	**Negative**	**Positive**	**Positive**	+++	+++	++	++	**Psychrotolerant**	++	++	+++

Table 1 (continued)

Number	Colony color*	Urease test**	DBB test***	Classification	Carbon source assimilation			Growth temperature					Metal tolerance		
					Methanol	Phenol	n-Hexadecane	5 °C	15 °C	25 °C	30 °C	Classification	$K_2Cr_2O_7$	$CdCl_2$	$CuSO_4$
296	**White**	**Variable**	**Negative**	**Ascomycetous**	**Negative**	**Positive**	**Positive**	+++	+++	+++	+++	**Psychrotolerant**	++	++	+++
297	White	Variable	Negative	ascomycetous	Negative	Negative	Positive	+++	+++	+++	+	Psychrotolerant	+	++	+++
300	Pink/orange	–	–	Basidiomycetous	Negative	Negative	Positive	+++	+++	+++	+++	Psychrotolerant	+	+	++++
303	White	Variable	Negative	Ascomycetous	Negative	Negative	Positive	++	+++	–	–	Psychrophilic	–	–	++
304	White	Variable	Negative	Ascomycetous	Positive	Negative	Positive	++	+++	–	–	Psychrophilic	–	–	++
305	Pink/orange	–	–	Basidiomycetous	Negative	Negative	Positive	++	+++	++	++	Psychrotolerant	++	+	+++
307	White	Positive	Positive	Basidiomycetous	Negative	Negative	Positive	++	+++	+	+	Psychrotolerant	–	–	+++
308	Pink/orange	–	–	Basidiomycetous	Negative	Negative	Positive	+	+++	+	+++	Psychrotolerant	++	–	++
309	Pink/orange	–	–	Basidiomycetous	Negative	Negative	Positive	+++	+++	+++	+	Psychrotolerant	+++	+	++
313	White	Negative	–	ascomycetous	Negative	Negative	Positive	+++	+++	+++	–	Psychrophilic	+	++	+++
314	**White**	**Negative**	–	**Ascomycetous**	**Negative**	**Positive**	**Positive**	+++	+++	+++	+++	**Psychrotolerant**	–	–	++
318	White	Negative	–	Ascomycetous	Negative	Negative	Positive	+++	+++	+++	++	Psychrotolerant	+	++	+++
322	White	Positive	Positive	Basidiomycetous	Negative	Negative	Positive	++	+++	+	–	Psychrophilic	–	–	–
330	White	Positive	Positive	Basidiomycetous	Negative	Negative	Positive	+++	+++	+	+++	Psychrotolerant	+++	+++	+++
337	Pink/orange	–	–	Basidiomycetous	Negative	Negative	Positive	+++	+++	–	–	Psychrophilic	+	–	+
340	Pink/orange	–	–	basidiomycetous	Negative	Negative	Positive	++	+++	+	+	Psychrotolerant	–	++	++
341	Pink/orange	–	–	Basidiomycetous	Negative	Negative	Positive	+++	+++	+++	+	Psychrotolerant	+	+++	+++
346	White	Positive	Negative	Ascomycetous	Negative	Negative	Positive	+++	+++	+++	+	Psychrotolerant	+	++	+++
349	Pink/orange	–	–	Basidiomycetous	Negative	Negative	Positive	+++	+++	+	+	Psychrotolerant	–	++	+++
356	White	Positive	Positive	Basidiomycetous	Negative	Negative	Positive	–	+++	+	+	psychrotolerant	–	–	–
357	Pink/orange	–	–	Basidiomycetous	Negative	Negative	Positive	+++	+++	+++	–	Psychrophilic	–	+++	+++
358	White	Positive	Positive	Basidiomycetous	Positive	Negative	Negative	++	+++	++	+++	Psychrotolerant	+	+	+

Those isolates in bold letter were selected for molecular identification

"–"No growth, "+" minimal growth (colonies barely visible, of around 1 mm), "++" moderate growth (colonies of approximately 2 mm), "+++" abundant growth (colonies of approximately 4 mm)

*If the isolate presented colony pigmentation, it was considered as basidiomycetous

**If urease test was negative not further test was performed

***DBB tests was performed in isolates with positive and variable urease test results

Table 2 Molecular identification of selected yeast isolates

Isolate	NCBI	Identity	Accession number	AFTOL	Identity	Accession number	Selection
6	*Cryptococcus victoriae* 26S ribosomal RNA gene, partial sequence	99	*AF363647.1*	*Cryptococcus* sp	98	*719*	*Cryptococcus victoriae*
9	*Cryptococcus gastricus 26S ribosomal RNA gene, partial sequence*	99	*AF137600.1*	*Cryptococcus* sp	98	*719*	*Cryptococcus victoriae*
12	*Cryptococcus terricolus* strain CBS4517 26S ribosomal RNA gene, partial sequence	99	*AF181520.1*	*Cryptococcus gastricus*	94	*1887*	*Cryptococcus terricolus*
13	*Meyerozyma guilliermondii* NRRL Y-2075 28S rRNA, partial sequence; from TYPE material	99	*NG_042640.1*	*Candida guilliermondii*	98	*1270*	*Meyerozyma guillermondii*
37	*Guehomyces pullulans* isolate AFTOL-ID 1958 25S large subunit ribosomal RNA gene, partial sequence	99	*EF551318.1*	*Guehomyces pullulans*	100	*1958*	*Guehomyces pulluans*
53	*Guehomyces pullulans* isolate AFTOL-ID 1958 25S large subunit ribosomal RNA gene, partial sequence	99	*EF551318.1*	*Guehomyces pullulans*	98	*1958*	*Guehomyces pullulans*
54	*Fellomyces penicillatus* 26S ribosomal RNA gene, partial sequence	99	*AF177405.1*	*Tremella globispora*	90	*1959*	*Fellomyces penicillatus*
59	*Meyerozyma guilliermondii* NRRL Y-2075 28S rRNA, partial sequence; from TYPE material	99	*NG_042640.1*	*Candida guilliermondii*	98	*1270*	*Meyerozyma guillermondii*
84	*Cryptococcus gastricus* 26S ribosomal RNA gene, partial sequence	99	*AF137600.1*	*Cryptococcus gastricus*	99	*1887*	*Cryptococcus gastricus*
99	*Metschnikowia australis* voucher 1.AURO 26S ribosomal RNA gene, partial sequence*	99	*KT970742.1*	*Metschnikowia bicuspidata*	94	*1326*	*Metschnikowia* sp
134	*Cryptococcus watticus* 26S ribosomal RNA gene, partial sequence*	99	*AY138478.1*	*Trichosporon lignicola*	89	*1802*	*Cryptococcus* sp
166	*Phenoliferia glacialis* strain A19 26S ribosomal RNA gene, partial sequence	99	*EF151258.1*	*Kriegeria eriophori*	98	*886*	*Phenoliferia glacialis*
171	*Pichia caribbica* strain NRRL Y-27274 26S ribosomal RNA gene, partial sequence	99	*EU348786.1*	*Candida guilliermondii*	98	*1270*	*Pichia caribbica*
172	*Rhodotorula muscorum* 26S ribosomal RNA gene, partial sequence	99	*AF070433.1*	*Leucosporidium scottii*	98	*718*	*Rhodotorula muscorum*
174	*Rhodotorula muscorum* 26S ribosomal RNA gene, partial sequence	99	*AF070433.1*	*Leucosporidium scottii*	98	*718*	*Rhodotorula muscorum*

Table 2 (continued)

Isolate	NCBI	Identity	Accession number	AFTOL	Identity	Accession number	Selection
190	*Mrakia frigida* CBS 5270 28S rRNA, partial sequence; from TYPE material	99	*NG_042346.1*	*Mrakia frigida*	99	*1818*	*Mrakia frigida*
197	*Phenoliferia glacialis* strain A19 26S ribosomal RNA gene, partial sequence	99	*EF151258.1*	*Kriegeria eriophori*	97	*886*	*Phenoliferia glacialis*
210	*Pichia caribbica* strain NRRL Y-27274 26S ribosomal RNA gene, partial sequence	99	*EU348786.1*	*Candida guilliermondii*	98	*1270*	*Pichia caribbica*
211	*Phenoliferia glacialis*-strain DBVPG 5917 26S ribosomal RNA gene, partial sequence*	96	*KC433875.1*	*Kriegeria eriophori*	95	*886*	*Basidiomycetous yeast*
217	*Cystobasidium laryngis* strain CBS 2221 26S ribosomal RNA gene, partial sequence	99	*AF189937.1*	*Occultifur externus*	93	*860*	*Cystobasidium laryngis*
235	*Candida smithsonii* strain BG02-7-13-007B-1-2 26S ribosomal RNA gene, partial sequence	99	*AY518525.1*	*Candida guilliermondii*	98	*1270*	*Candida smithsonii*
243	*Cryptococcus victoriae* 26S ribosomal RNA gene, partial sequence	99	*AF363647.1*	*Cryptococcus* sp	98	*719*	*Cryptococcus victoriae*
248	*Rhodotorula mucilaginosa* 26S ribosomal RNA gene, partial sequence	100	*AF070432.1*	*Rhodotorula mucilaginosa*	100	*1548*	*Rhodotorula mucilaginosa*
249	*Metschnikowia australis* voucher 1. AURO 26S ribosomal RNA gene, partial sequence*	99	*KT970742.1*	*Metschnikowia bicuspidata*	95	*1326*	*Metschnikowia* sp
251	*Cryptococcus victoriae* 26S ribosomal RNA gene, partial sequence	99	*AF363647.1*	*Cryptococcus* sp	98	*719*	*Cryptococcus victoriae*
252	*Metschnikowia australis* voucher 1. AURO 26S ribosomal RNA gene, partial sequence	99	*KT970742.1*	*Metschnikowia bicuspidata*	95	*1326*	*Metschnikowia* sp
257	*Rhodotorula fragaria* 26S ribosomal RNA gene, partial sequence	99	*AF070428.1*	*Leucosporidium scottii*	98	*718*	*Rhodotorula fragaria*
264	*Mrakia frigida* CBS 5270 28S rRNA, partial sequence; from TYPE material	99	*NG_042346.1*	*Mrakia frigida*	99	*1818*	*Mrakia frigida*
265	*Phenoliferia glacialis strain A19 26S ribosomal RNA gene, partial sequence*	99	*EF151258.1*	*Kriegeria eriophori*	98	*886*	*Phenoliferia glacialis*
273	*Leucosporidium creatinivorum* CBS 8620 28S rRNA, partial sequence; from TYPE material	100	*NG_042375.1*	*Leucosporidium scottii*	99	*718*	*Leucosporidium creatinivorum*

Table 2 (continued)

Isolate	NCBI	Identity	Accession number	AFTOL	Identity	Accession number	Selection
276	*Leucosporidium creatinivorum CBS 8620 28S rRNA, partial sequence; from TYPE material*	99	*NG_042375.1*	*Leucosporidium scottii*	99	*718*	*Leucosporidium creatinivorum*
279	*Rhodotorula mucilaginosa* 26S ribosomal RNA gene, partial sequence	99	*AF070432.1*	*Rhodotorula mucilaginosa*	100	*1548*	*Rhodotorula mucilaginosa*
293	*Cryptococcus victoriae* 26S ribosomal RNA gene, partial sequence	99	*AF363647.1*	*Cryptococcus* sp	97	*719*	*Cryptococcus victoriae*
296	*Nadsonia commutata* strain NRRL Y-7950 26S ribosomal RNA gene, partial sequence	100	*KC254858.1*	*Candida guilliermondii*	93	*1270*	*Nadsonia commutata*
314	*Nadsonia commutata* strain NRRL Y-7950 26S ribosomal RNA gene, partial sequence	99	*KC254858.1*	*Candida guilliermondii*	93	*1270*	*Nadsonia commutata*

*Sequences blasted against non-type material

effect on both physiological activities of plants and human health (Suciu et al. 2008). Part of the industrial plants generating phenol-rich effluents also discharge heavy metals, resulting in growth inhibition of most phenol-degrading microorganisms used for wastewater disposal (Thavamani et al. 2012; Wong et al. 2015). Thus, much attention should be paid to the phenol removal performance of microorganisms in media with the presence of heavy metals ions. It was found from the previous study that bacterial strains *Pseudomonas rhodesiae* and *Bacillus subtilis* could remove phenol and survive in heavy metal polluted environment (Satchanska et al. 2015). Regarding fungi, several reports have mentioned their resistance to metal ions (Fernández et al. 2013). However, no evaluation of heavy metal effect on phenol biodegradation by Antarctic isolations has previously been performed. In this study, 24 of the 128 isolates (19%) exhibited some degree of tolerance to the three studied metals and can use phenol as carbon source. These strains, mainly those showing high levels of metal tolerance (as strains 190 and 276), represents promising strains for using in low temperature treatment of effluents containing phenol and high levels of metals ions.

Effect of temperature on growth of yeasts

All strains grew in complex medium (YM) at temperatures ranging from 5 to 25 °C. The bulk of the isolates was psychrotolerant, but the true psychrophiles, showing no growth above 20 °C (Morita 1975) represented 41% ($n=53$) of the isolates. The predominance of psychrotolerant fungi in cold environments has been previously noted, and is attributable to seasonal and local increases in soil temperature due to insolation (Robinson et al. 2001) mainly when samples were taken from surface soils and other sites directly exposed to solar irradiance. In our study, the temperature measured in situ at the different sampling sites ranged from 0 to 10 °C, but because of global warming and climate change, higher temperatures have been reported in this region (Royles et al. 2013). Peck et al. (2007) showed that different sites at 25 de Mayo Island present temperatures in the range 2.8–11.6 °C.

Identification of the selected isolates

Among the isolates which could assimilate phenol as carbon source, we selected those showing tolerance to more than one of the heavy metal ions tested (Cr, Cd, and Cu) for identification (Table 1).

Two different data bases were used, NCBI and AFTOL. As was mentioned above, sequences showing 97–98% identity were tentatively identified to the genus level. Sequences showing less than 97% identity were considered unidentified. With sequences showing identity values lesser than 97% compared with type strains, a new blast using non-type material was performed to identify our sequences at genus level or, at least, define each one as a sequence coming from an asco- or basidiomycetous yeast.

Based on the literature, yeasts living in Antarctic and sub-Antarctic maritime and terrestrial habitats belong mainly to the *Cryptococcus, Candida, Rhodotorula*, and

Mrakia genera (Buzzini et al. 2012; Carrasco et al. 2012; Rovati et al. 2013). *Cryptococcu*s spp. has been isolated repeatedly from soil samples, and some researchers have described them as the most important life form in Antarctic desert soils (Vishniac and Kilinger 1986). In fact, many new species of the genus *Cryptococcus* have been obtained from Antarctic environments (Scorzetti et al. 2000; Guffogg et al. 2004; Zhang et al. 2014). Others genera (approximately 53) have been reported for Antarctica but in smaller proportions (Thomas-Hall et al. 2010; Carrasco et al. 2012; Alcaíno et al. 2015).

In this work, not only these four genera were isolated but also *Candida, Cistobasidium, Fellomyces, Guehomyces, Leucosporidium, Metschnikowia, Meyerozyma, Nadsonia, Phenolifera*, and *Pichia*.

The isolates belong to both asco- and basidiomycetous genera, which confirmed that the use of a media with low concentration of nutrients stimulated the isolation of more diverse genera, which contributes in a great extent to the knowledge of fungi biodiversity from this cold and isolated region of the world. In addition, up to the moment of publication of this work, some species here identified was not previously reported for the Antarctic continent, such as *Candida smithsonii* and *Pichia caribbica*.

Conclusion

One hundred and twenty-eight yeast isolates have been obtained from Antarctica and were tested for pollutant assimilation and heavy metal ions tolerance. The identified yeasts belong to widely reported, cold-adapted yeast taxa, most of them included into oligotrophic, slow-growing, and metabolically diverse basidiomycetous genera.

The prevalence of basidiomycetous yeast in Antarctic samples remains unclear, but could be related to the oligotrophy of soil and the most water samples and, also to the isolation scheme employed. As previously emphasized, oligotrophic microorganisms are usually related to the ability to degrade a broad spectrum of substrates, whilst copiotrophic microorganisms are related to the efficient degradation of easily accessible substrates.

Despite the genus of yeasts isolated from cold environments, research in the field of cold-adapted yeasts is relatively young. It is generally accepted that information regarding cold-adapted yeasts will have a continuous increase, especially with the development of new microbiological and molecular methodologies.

The present study is the first report proving a high tolerance of some cold-adapted Antarctic yeasts isolates towards high concentrations of heavy metal salts and phenol as a carbon source. The tolerance to heavy metals ions of the phenol-degrading cold-adapted yeasts evidences that the strains might be promising in treating some kinds of phenol-polluted industrial wastewater containing heavy metals, such as effluents from petroleum refineries. The data and results presented in this work open new avenues to explore the cold-tolerant yeasts isolated from Antarctica, providing information for its use as tools in bioremediation processes at low temperatures and also giving data regarding their possible ecological role under such extreme conditions.

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