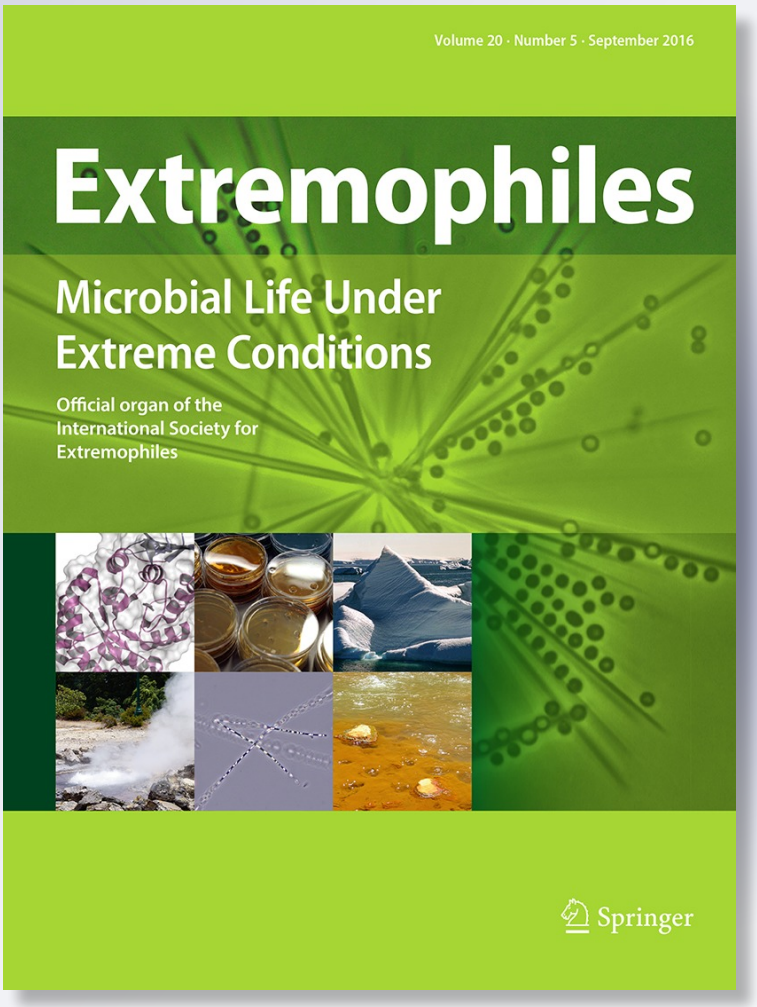
Yeasts from sub-Antarctic region: biodiversity, enzymatic activities and their potential as oleaginous microorganisms

A. Martinez, I. Cavello, G. Garmendia, C. Rufo, S. Cavalitto & S. Vero

Extremophiles
Microbial Life Under Extreme Conditions

ISSN 1431-0651
Volume 20
Number 5

Extremophiles (2016) 20:759-769
DOI 10.1007/s00792-016-0865-3



Springer

Extremophiles (2016) 20:759–769
DOI 10.1007/s00792-016-0865-3

ORIGINAL PAPER

Yeasts from sub-Antarctic region: biodiversity, enzymatic activities and their potential as oleaginous microorganisms

A. Martinez[1] · I. Cavello[2] · G. Garmendia[1] · C. Rufo[3] · S. Cavalitto[2] · S. Vero[1]

Received: 1 April 2016 / Accepted: 12 July 2016 / Published online: 28 July 2016

Abstract Various microbial groups are well known to produce a range of extracellular enzymes and other secondary metabolites. However, the occurrence and importance of investment in such activities have received relatively limited attention in studies of Antarctic soil microbiota. Sixty-one yeasts strains were isolated from King George Island, Antarctica which were characterized physiologically and identified at the molecular level using the D1/D2 region of rDNA. Fifty-eight yeasts (belonging to the genera *Cryptococcus, Leucosporidiella, Rhodotorula, Guehomyces, Candida, Metschnikowia* and *Debaryomyces*) were screened for extracellular amylolytic, proteolytic, esterasic, pectinolytic, inulolytic xylanolytic and cellulolytic activities at low and moderate temperatures. Esterase activity was the most common enzymatic activity expressed by the yeast isolates regardless the assay temperature and inulinase was the second most common enzymatic activity. No cellulolytic activity was detected. One yeast identified as *Guehomyces pullulans* (8E) showed significant activity across six of seven enzymes types tested. Twenty-eight yeast isolates were classified as oleaginous, being the isolate 8E the strain that accumulated the highest levels of saponifiable lipids (42 %).

Keywords Bioenergetics · Enzymes · Psychrophiles

Communicated by F. Robb.

✉ S. Vero
svero@fq.edu.uy

[1] Cátedra de Microbiología, Departamento de Biociencias, Facultad de Química, Universidad de la República, Montevideo, Uruguay

[2] Research and Development Center for Industrial Fermentations, CINDEFI (CONICET, La Plata, UNLP), Calle 47 y 115 (B1900ASH), La Plata, Argentina

[3] Facultad de Química, Instituto Polo Tecnológico, Universidad de la República, By Pass Ruta 8 s/n, Pando, Canelones, Uruguay

Introduction

Extremely cold environments (i.e., Arctic and Antarctic regions and European glaciers) can be successfully colonized by a group of extremophilic microorganisms known as psychrophiles. This group of microorganisms is defined by an optimum growth temperature of 15 °C, a maximum growth temperature below 20 °C, and a minimum growth temperature equal to or below 0 °C (Morita 1975). However, in those extreme environments, psychrotrophic or psychrotolerant microorganisms, which are cold-adapted mesophiles, also exist, and compete well for resources (Mohamed Hatha et al. 2013). Such microorganisms have developed strategies to protect themselves against environmental and nutritional stress of extremely cold regions and to resist seasonal climate changes which sometimes constitute an advantage over obligate psychrophiles.

Psychrotolerant yeasts are increasingly attracting attention for their enormous biotechnological potential (Buzzini and Margesin 2014), including the production of cold-active enzymes, such as amylases, cellulases, invertases, inulinases, proteases, lipases which are currently broadly used in food, biofuel and detergent industries (Margesin and Miteva 2011). Such enzymes could also be produced by obligate psychrophiles. However, since psychrophilic yeasts require refrigerated conditions to grow, costs associated to their production would be higher than those involved in the culture of psychrotrophes, especially in large scale producing processes.

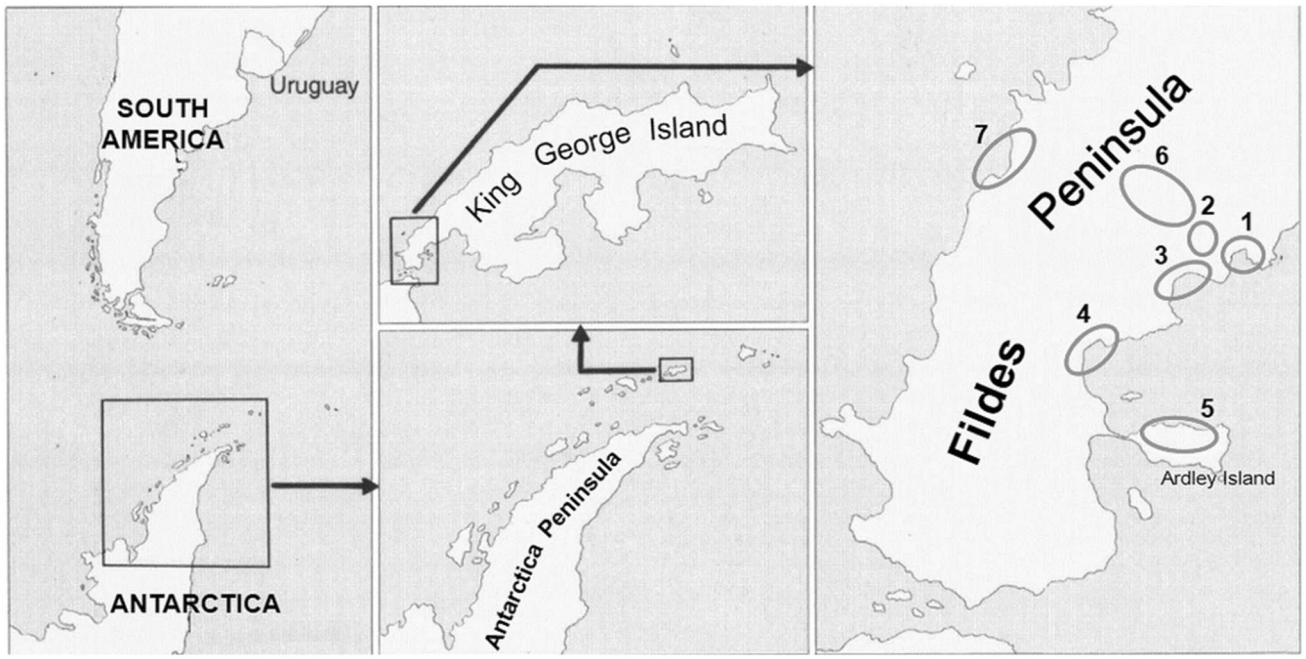


Fig. 1 Sample site locations on King George Island. *1* Collins Bay, *2* Uruguay Lake, *3* Norme Cove, *4* Ardley Cove, *5* Ardley Island, *6* Surrounding Collins glacier and *7* Drake Bay

Psychrotrophic yeasts have also been explored as oleaginous microorganisms to be used as a source of lipids for biodiesel production (Rossi et al. 2009). In recent years there has been a significant amount of work on oleaginous yeasts (Pereyra et al. 2014; Sitepu et al. 2014; Garay et al. 2016; Zhang et al. 2015) which have the ability to accumulate at least 20 % oil as a percentage of cell dry weight in appropriate culture conditions (Ratledge and Hall 1979). Intracellular lipids of oleaginous yeasts are mainly stored in the form of triglycerides which can be extracted and transformed in biodiesel after a transesterification process (Sitepu et al. 2014). It has been demonstrated that varying growth temperature changed the fatty acid composition of those stored lipids and, increased the content of unsaturated acids (Amaretti et al. 2010). In that context, oleaginous yeasts that could grow at low and moderate temperatures, such as psychrotrophic yeasts, would be good candidates to produce different types of biodiesel that could be used at different temperatures.

Although the first report of Antarctic yeasts was published 50 years ago (Di Menna 1966) current reports have mainly focused on cold-tolerant Bacteria and Archaea. Two extensive reviews about Antarctic yeast biodiversity and biotechnological applications have been published (Vishniac 2006b; Shivaji and Prasad 2009). However, Connell et al. (2010) reported that approximately 43 % of the Antarctic yeasts isolated by them were assigned to undescribed species, reflecting the lack of knowledge about cultivable yeasts that colonize Antarctic soils. King George Island is the largest island within the South Shetland Islands archipelago. As typical of this region of Antarctica, the island hosts a variety of terrestrial ecosystems and soil habitats, including pristine soils and vegetation, vertebrate-influenced habitats and sites under human impact (Tin et al. 2009).

This work describes the isolation of yeasts from terrestrial and maritime habitats of this island. Yeast isolates were characterized physiologically and identified at the molecular level by the analysis of the D1/D2 sequences. In addition, the biotechnological potential of the isolates through the ability to produce extracellular cold-adapted enzymes and intracellular lipids was evaluated. Characterization of the enzyme activities and the fatty acid composition of stored lipids may contribute to enhance the potential of the yeasts in industrial applications.

Materials and methods

Sampling sites

Psychrotrophic yeasts were isolated from soil and terrestrial and marine waters from different sites on the Fildes peninsula, King George Island (Lat 62°11′04S; Long 58°54″W), situated 120 km off the coast of the Antarctic Peninsula in the Southern Ocean. Samples were collected in January 2012 and March 2013 during expeditions organized by the Instituto Antartico Uruguayo. Sampling

sites are indicated in Fig. 1. Different numbers of samples (indicated in brackets) were taken from soil, sediments and water in the surroundings of Collins Bay (12), Norme Cove (11), Ardley Island (16), Ardley Cove (3), Uruguay Lake (10), Drake Bay (14) and Collins Glacier (4). A total of 70 samples were collected in both years. In each case, geographical coordinates and temperature were registered. Samples were collected in sterile tubes of 50 ml and kept at 0 °C until they were processed.

Sample processing and yeast isolation

To isolate cold-adapted yeasts, 10 g of soil samples were suspended in 90 ml of sterile water and 100 µl of the obtained suspension and 1/10 and 1/100 dilutions were spread onto the surface of Dichloran Rose Bengala Chloramphenicol Agar (DRBCA) (Merk, Darmstadt, Germany) plates. In case of water samples, 100 µl of the sample and the corresponding dilutions were spread onto the same medium. Plates were incubated at 25 °C for 7 days. When no yeasts were recovered from a sample, an enrichment procedure was used. Briefly, ten grams or milliliters of each sample was inoculated in 90 ml of Yeast Nitrogen Base (YNB) (Difco, Sparks, USA) supplemented with 2 % glucose and chloramphenicol (100 µg/ml) and incubated for 7 days at 25 °C in an orbital shaker at 150 rpm. Yeasts obtained from those enrichment cultures were isolated on DRBCA plates. Yeast colonies obtained in all cases were examined macro and microscopically. Those showing a different morphotype were selected from each sample and purified by streaking them on Potato Dextrose Agar (PDA) (Oxoid, Hampshire, UK) plates at 25 °C. Additionally, the presence of obligate psychrophilic yeasts was evaluated in each sample by streaking 100 µl of liquid samples and soil suspensions onto DRBCA and incubated at 4 °C for 15 days. All different colonies were transferred to DRBCA plates and incubated at 25 °C for days. Those isolates that could grow at 4 °C but not at 25 °C were considered psychrophilic and kept to be identified. All isolates were preserved in 20 % glycerol at −70 °C.

Yeast identification

All isolates were identified at species level by sequence analysis of the D1/D2 variable domains at the 5′ end of the large subunit rRNA gene. DNA extraction was carried out as described by Schena et al. (1999). PCR fragments were generated using primers ITS1 and D2R covering both the ITS1–ITS2 and D1/D2 region. The thermal profile was 96 °C 2 min, followed by 35 cycles of 96 °C 30 s, 51 °C 45 s, 72 °C 120 s, and a final extension step at 72 °C for 7 min. Nucleotide sequences of the PCR products were determined in both directions at Macrogen (Macrogen Inc., Seoul, Korea). Sequences were aligned with MEGA version 5, visually corrected and compared to NCBI databases using BLAST. Phylogenetic analyses of D1/D2 sequences of the selected isolates were conducted using MEGA version 5. DNA sequences were aligned with sequences of homologous regions of closely related strains retrieved from GenBank. Two phylogenetic trees were constructed, one with ascomycetous and another with basidiomycetous species. In both cases, evolutionary distances were computed using the Jukes–Cantor method, and phylogenetic trees were obtained by neighbor-joining. All positions containing alignment gaps and missing data were eliminated in pairwise sequence comparisons (pairwise deletion option). Stability of clades was assessed with 1000 bootstrap replications.

Culturable yeast diversity

Diversity, richness and dominance of culturable yeasts, were quantified using Shannon, Margalef's and Simpson's indices, respectively, with the aid of the software PAST, version 3.11 (Hammer et al. 2001). Dominance was calculated as 1-Simpson index.

Determination of the effect of temperature on growth of yeast isolates

All the isolates were tested for the ability to grow at different temperatures (4, 20, and 28 °C) on PDA plates. The plates were inoculated with yeast cells grown for 24–48 h, and incubated at different temperatures. Growth was monitored visually on a daily basis for a week, except for the Petri dishes incubated at 4 °C, which were monitored for 2 weeks. Colony growth was assessed qualitatively (De García et al. 2007).

Production of hydrolytic enzymes

24–48 h cultures of the yeasts grown at 25 °C on PDA were used to inoculate Petri dishes containing the substrates selected. The inoculated dishes were incubated at low (8 °C) and room temperature (20 °C) and the expressions of extracellular enzymes were analyzed after 7 days. A full description of the protocols is provided above; they were carried out according Buzzini and Martini (2002).

The ability to degrade long-chain esters (esterasic activity) was detected using sorbitan monooleate (Tween 80). Tween 80 medium agar consisted of 10 g/l peptone, 5 g/l NaCl, 0.1 g/l $CaCl_2 \cdot 2H_2O$, 20 g/l agar (pH 7.0). The formation of a clear zone around a colony due to complete degradation of the substrate indicates esterasic activity.

Proteolytic activity was assessed using skim milk agar medium consisting of 10 g/l skim milk and 20 g/l agar (pH

6.6). After incubation, a positive reaction was detected as a clear zone around the colony in the opaque medium.

The ability to degrade long-chain esters (*esterasic activity*) was detected using sorbitan monooleate (Tween 80). Tween 80 medium agar consisted of 10 g/l peptone, 5 g/l NaCl, 0.1 g/l $CaCl_2{\cdot}2H_2O$, 20 g/l agar (pH 7.0). The formation of a clear zone around a colony due to complete degradation of the substrate indicates esterasic activity.

Proteolytic activity was assessed using skim milk agar medium consisting of 10 g/l skim milk and 20 g/l agar (pH 6.6). After incubation, a positive reaction was detected as a clear zone around the colony in the opaque medium.

The ability to degrade starch (*amylolytic activity*) and inulin (*inulinase activity*) was evaluated using starch or inulin agar, which consisted of 10 g/l soluble starch or inulin, 2 g/l yeast extract, 5 g/l peptone, 0.5 g/l $MgSO_4$, 0.5 g/l NaCl, 0.15 g/l $CaCl_2$ and 20 g/l agar (pH 6.0). After incubation, the plates were flooded with Lugol's iodine solution. A pale yellow zone around the colony indicates starch or inulin degradation.

To detect *pectinases enzymes,* a selective medium containing 10 g/l pectin, 1.4 g/l NH_4SO_4, 2.0 g/l K_2HPO_4, 0.2 g/l $MgSO_4{\cdot}7H_2O$, 1 ml sol. A (5 mg/l $FeSO_4{\cdot}H_2O$, 1.6 mg/l $MnSO_4{\cdot}H_20$, 2 mg/l $CoCl_2$) and 20 g/l agar was used. Pectinase producing colonies was screened and selected by flooding the solid media plates with Lugol's iodine solution.

Screening of *cellulase activity* was done on CMC agar medium containing 2.0 g/l $NaNO_3$, 1.0 g/l K_2HPO_4, 0.5 g/l $MgSO_4$, 0.5 g/l KCl, 2.0 g/l carboxymethylcellulose (CMC) sodium salt, 0.2 g/l peptone and 17 g/l agar. Detection of cellulose activity was done by Congo Red solution (0.1 %) and destained with NaCl 1 M.

To detect *xylanases*, xylan agar medium containing 10 g/l xylan, 5.0 g/l yeast extract, 5 g/l peptone, 1 g/l K_2HPO_4, 0.2 g/l $MgSO_4{\cdot}7H_2O$ and 20 g/l agar was used. Xylanase producing colonies was screened and selected by flooding the solid media plates with Congo Red solution (0.1 %) and destained with NaCl 1 M.

Screening of oleaginous yeasts

Each yeast strain was grown at 20 °C in a rotary shaker at 150 rpm in 250 ml Erlenmeyer flasks containing 50 ml of a liquid nitrogen-limited medium developed by Thakur et al. (1989) for intracellular lipid accumulation. The medium consisted of (g l^{-1}): glucose, 40; KH_2PO_4, 0.75; yeast extract, 1.5; NH_4NO_3, 0.285; $CaCI_2{\cdot}2H_20$, 0.4; $MgSO_4{\cdot}7H_20$, 0.4 (Thakur et al. 1989). The pH of the medium was initially adjusted to 5.0. Each flask was inoculated with five ml of a yeast suspension (turbidity = McFarland 1) prepared in sterile distilled water from a 48 h culture at 25 °C. After 5 days of incubation, cultures were centrifuged and pellets were washed and then resuspended in sterile distilled water to obtain a cell suspension with a concentration that, when it was diluted 40 times, had an OD 600 nm = 0.6. A strain of *Saccharomyces cerevisiae* obtained from an active dry yeast commercial product (Fleischman®) was included in the assay as non-oleaginous yeast (Vorapreeda et al. 2012).

The intracellular lipid content was estimated as described by Sitepu et al. (2012) with some modifications. In each case, 160 µl of yeast suspension, prepared as described above, was added to 800 µl of DMSO (dimethyl sulfoxide) dissolved in 2240 µl of distilled water. A volume of 14.72 µl of 0.1 mg/ml solution of Nile red in acetone was added to the mixture and after 2 min in the dark, fluorescence was determined in a Shimadzu—RF-5301PC spectrophotometer. Excitation wavelength was of 488 nm and fluorescence of the sample was determined as the maximum value obtained in an emission wavelength spectra from 400 to 700 nm (Kimura et al. 2004).

The content of saponifiable lipids, of some strains, was quantified as described by Pereyra et al. (2014) by gravimetric determination after chemical extraction. Briefly, biomass from 40 ml of each culture was washed with distilled water and then treated with 5 ml of KOH 30 % and 5 ml of ethanol 95 %. The mixture was incubated overnight at 70 °C and then unsaponifiable matter was removed with hexane. The pH of the remaining aqueous phase was adjusted to 1 with HCl, and then fatty acids were extracted twice with 10 ml of hexane. After hexane evaporation under reduced pressure, the solid residue obtained was weighed. At the same time, in each case, biomass of yeasts was determined as dry weight of the cells contained in 2 ml of culture. Amounts of intracellular saponifiable lipids were expressed as percentage of dry mass corresponding to the fatty acids obtained as described above. The levels of intracellular saponifiable lipids were determined for the strain that showed the highest level of fluorescence, for *Saccharomyces cerevisiae* commercial strain as non-oleaginous yeast and for a strain which produced an intermediate amount of fluorescence (C. sake 41E).

Two replicates per treatment were performed. Data were subjected to analysis of variance and where the F ratio was significant ($P \leq 0.05$), differences among treatment means were separated using Fischer's least significant difference using Infostat (Di Rienzo et al. 2009).

Results

Yeast occurrence

Two-thirds ($n = 43$) of all samples collected during the Antarctic expeditions were solid samples from soil or

Table 1 Identification, source and growth temperature range of yeast isolates

Strain	Source	4 °C	20 °C	28 °C	Yeast identification
4E	Soil	+	+	+	*Cryptococcus aerius*
4BE	Soil	+	+	−	*Cryptococcus keelungensis*
4CE	Soil	+	+	+	*Cryptococcus aerius*
5E	Soil	+	+	−	*Leucosporidiella fragaria*
7AE	Soil	+	+	+	*Rhodotorula laryngis*
7BE	Soil	−	+	−	*Candida davisiana*
8E	Soil	+	+	+	*Guehomyces pullulans*
10E	Soil	+	+	+	*Cryptococcus victoriae*
12R	Soil	+	+	+	*Candida glaebosa*
12D	Soil	+	+	+	*Candida glaebosa*
13R	Soil	+	+	−	*Leucosporidiella fragaria*
14E	Water	+	+	+	*Cryptococcus victoriae*
14BE	Water	+	+	+	*Candida glaebosa*
15E	Soil	+	+	+	*Candida glaebosa*
17E	Soil	+	+	+	*Candida glaebosa*
19R	Soil	+	+	−	*Cryptococcus victoriae*
19DA	Soil	+	+	+	*Cryptococcus victoriae*
19DB	Soil	+	+	+	*Leucosporidiella fragaria*
20E	Water	+	+	−	*Metschnikowia australis*
21R	Soil	+	+	+	*Candida glaebosa*
22AE	Soil	+	+	+	*Cryptococcus gastricus*
22BE	Soil	+	+	−	*Cryptococcus gilvescens*
24E	Soil	+	+	+	*Candida glaebosa*
29AE	Water	+	+	+	*Rhodotorula mucilaginosa*
29BE	Water	+	+	+	*Debaryomyces hansenii*
32R	Soil	+	+	−	*Metschnikowia australis*
33AE	Water	+	+	+	*Rhodotorula laryngis*
35E	Water	+	+	+	*Debaryomyces hansenii*
36E	Soil	+	+	+	*Cryptococcus albidosimilis*
37E	Water	+	+	+	*Cryptococcus tephrensis*
37BE	Water	+	+	+	*Metschnikowia australis*
39E	Water	−	+	−	*Metschnikowia australis*
41E	Soil	+	+	+	*Candida sake*
A3	Water	+	−	−	*Mrakia frigida*
F2	Water	+	−	−	*Phenoliferia psychrophenolica*
F3A	Soil	+	+	−	*Candida davisiana*
F3B	Soil	+	+	+	*Rhodotorula laryngis*
F3C	Soil	+	+	−	*Candida davisiana*
F3E	Soil	−	+	+	*Rhodotorula minuta*
F4A	Soil	+	+	+	*Rhodotorula laryngis*
F6E	Soil	+	+	+	*Debaryomyces hansenii*
F7A	Soil	+	+	+	*Debaryomyces hansenii*
F9D	Soil	+	+	+	*Debaryomyces hansenii*
F11	Water	+	−	−	*Glaciozyma antarctica*
F9H	Soil	+	+	+	*Debaryomyces hansenii*
F12B	Soil	+	+	+	*Debaryomyces hansenii*
F12C	Soil	+	+	−	*Leucosporidiella fragaria*
F12D	Soil	+	+	+	*Rhodotorula laryngis*
F13D	Water	+	+	+	*Debaryomyces hansenii*
F13E	Water	+	+	+	*Cystofilobasidium infirmominiatum*
F18A	Water	+	+	+	*Debaryomyces hansenii*
F20A	Soil	+	+	−	*Leucosporidiella muscorum*
F22B	Water	+	+	+	*Candida zeylanoides*
F24A	Water	+	+	+	*Debaryomyces macquariensis*
F26A	Soil	+	+	+	*Rhodotorula laryngis*
F26D	Soil	+	+	+	*Cryptococcus victoriae*
F27A	Soil	+	+	−	*Metschnikowia australis*
F32A	Soil	+	+	+	*Debaryomyces hansenii*
F33B	Water	+	+	+	*Rhodotorula laryngis*
F36A	Water	+	+	+	*Candida sake*
F39A	Soil	+	+	+	*Debaryomyces hansenii*

sediments, while the rest ($n = 27$) were from terrestrial and marine waters. Temperatures of sampling sites varied from −0.4 to 11 °C. Psychrotrophic yeasts could not be isolated from all the samples. Moreover, in most cases (64 %) yeasts could not be recovered directly from samples, without a previous enrichment, showing that in those cases yeast concentration was lower than 10 cfu g^{-1} or 1 cfu ml^{-1} in solid and water samples, respectively. After an enrichment of 10 g or 10 ml of samples, yeasts could be recovered only from 60 % of the processed samples, which means that in 18 samples the concentration of culturable psychrotolerant yeasts was lower than 1 cfu in 10 g or 10 ml. In fact, yeast counts in all samples were low, being 5×10^4 cfu g^{-1} the highest concentration detected, which corresponded to a soil sample from Ardley Island (S62°12′45″; W58°55′16.9″). Obligate psychrophilic yeasts were isolated only from 3 samples of water collected at Collins Cove (F2), Ardley Island (F11) and Drake Bay (A13), as in shown in Table 1.

Yeast identification

Sixty-one yeast isolates were obtained. All of them could be identified to species level by molecular analysis, since in all cases, D1/D2 sequences showed more than 99 % homology with the corresponding to type strains of unique species. Relatedness of sequences of all isolates and those corresponding to the most closely related type strains retrieved from GenBank, is presented in the phylogenetic trees shown in Fig. 2.

Psychrotrophic isolates could be classified into eight genera (*Cryptococcus, Leucosporidiella, Rhodotorula, Guehomyces, Candida, Metschnikowia, Cystofilobasidium*, and *Debaryomyces*). Most of the isolates were identified as

basidiomycetous anamorphs, being *Cryptococcus* (12) and *Rhodotorula* (9) the most represented genera. Among phylum Ascomycota, yeasts isolates were affiliated to genera *Candida, Metschnikowia* and *Debaryomyces*. A total of 11 yeasts species were identified and *Debaryomyces hansenii* was the most abundant specie found (Fig. 2, phylogenetic analysis). The nucleotide sequences corresponding to the D1/D2 region of all the isolates have been deposited in GenBank under the accession numbers KU659485–KU659542. Psychrophilic isolates were identified as *Glaciozyma antarctica* (F11), *Phenoliferia psychrophenolica* (synonymy of *Rhodotorula psychrophenolic*) (F2) and *Mrakia frigida* (A13) and their sequences were also deposited in GenBank (Accession numbers KU883220, KU883219, KU883218, respectively).

Culturable yeast diversity

In general, the fungal community showed moderate diversity (Shannon index = 2.78) high richness (Margalef = 5.59) and low dominance (Simpson = 0.915, Dominance = 0.085) indices.

Effect of temperature on yeasts growth

All the yeast strains were able to grow at 20 °C. Three strains identified as *Candida davisiana* (7BE), *Metschnikowia australis* (39E) and *Rhodotorula minuta* (F3E) did not grow at 4 °C. On the other hand almost 76 % of the isolates could grow at 28 °C (Table 1).

Screening of enzymes activities at low and moderate temperatures

The ability of yeast isolates to produce hydrolytic enzymes was studied at 8 and 20 °C and the results are presented in Table 2. Fifty-two percent of the strains showed at least one extracellular enzyme activity at either 8 or 20 °C. 15 isolates exhibited 2–4 enzyme activities, being an exceptional isolated that exhibited 6 enzyme activities: *Guehomyces pullulans*. This yeast expressed different enzymatic profile when it was incubated at 8 or 20 °C. At 8 °C exhibited proteolytic, esterasic, amylolytic, pectinolytic and inulolytic activities while at 20 °C exhibited proteolytic, amylolytic, inulolytic, pectinolytic and xylanolytic activities. The most common enzymatic activity in the yeast isolates were esterase, regardless of the temperature at which it was tested. The second most common activity found was inulinase; 12 isolates were positive for this trait at 20 °C, while 7 isolates retained this activity at 8 °C. Amylolytic, xylanolytic and pectinolytic activities were less frequent, and were expressed in about 6–10 % of the strains. None of the strains was able to produce cellulose activity at either of the assessed temperatures. In some cases enzymatic activities were strain specific. Four isolates molecularly identified as *Metschniskowia australis* (20E, 32R, 37E and 39E) showed differences in their enzyme activity profile, as was also observed for the isolates identified as *C. victoriae* (4E, 10E, 14BE, 19R and 19DA) (Table 2). *Candida sake, Candida zeylanoides, Cryptococcus albidosimilis, Debaryomyces macquariensis, Rhodotorula minuta* and *Rhodotorula mucilaginosa* were not able to hydrolyze any of the compounds tested. Differences in the expression of extracellular enzymatic activities at 8 and 20 °C were observed. Two of seven tested activities showed a higher number of positive strains at 8 °C than at 20 °C. The biggest differences were observed for proteolytic activity; 18 strains were able to hydrolyze skim milk at 8 °C, whereas only 6 strains retain this activity at 20 °C.

Screening of oleaginous yeasts

The *Saccharomyces cerevisiae* strain used in this study accumulated saponifiable lipids representing 17 % of its dry biomass, as determined by gravimetry after chemical extraction, and exhibited 335.5 fluorescence units when exposed to Nile Red. Twenty-eight isolates showed not different or significantly lower levels of fluorescence than the *S. cerevisiae* strain, therefore they were considered as non-oleaginous yeasts. The strain 41E identified as *Candida sake*, which showed intermediate level of fluorescence, accumulated saponifiable lipids that represented the 24 % of its dry biomass and so it could be considered as an oleaginous strain. Thirty strains showed fluorescence values equal to or higher than this strain and could be considered as oleaginous microorganisms. *Guehomyces pullulans* (8E) showed the highest fluorescence values, which corresponded to an accumulation of 42 % of saponifiable lipids in its dry biomass (Fig. 3). Figure 3 shows the units of fluorescence when oleaginous yeasts (30) were exposed to Nile red in comparison to *Saccharomyces cerevisiae* and three yeast strains classified as non-oleaginous. The remaining yeast isolates showed fluorescence values significantly lower than *Saccharomyces cerevisiae* (data not shown)

Discussion

In this work, cold-adapted yeasts were isolated from terrestrial and marine samples from King George Island, in the sub-antarctic region. Both psychrotrophic and psychrophilic yeasts were found, being psychrotrophes markedly prevalent. Many previous reports about mycoflora from Antarctica also confirmed the prevalence of psychrotolerant yeasts and molds over obligate psychrophiles. According to Ruisi et al. (2007) psychrotolerant yeasts would

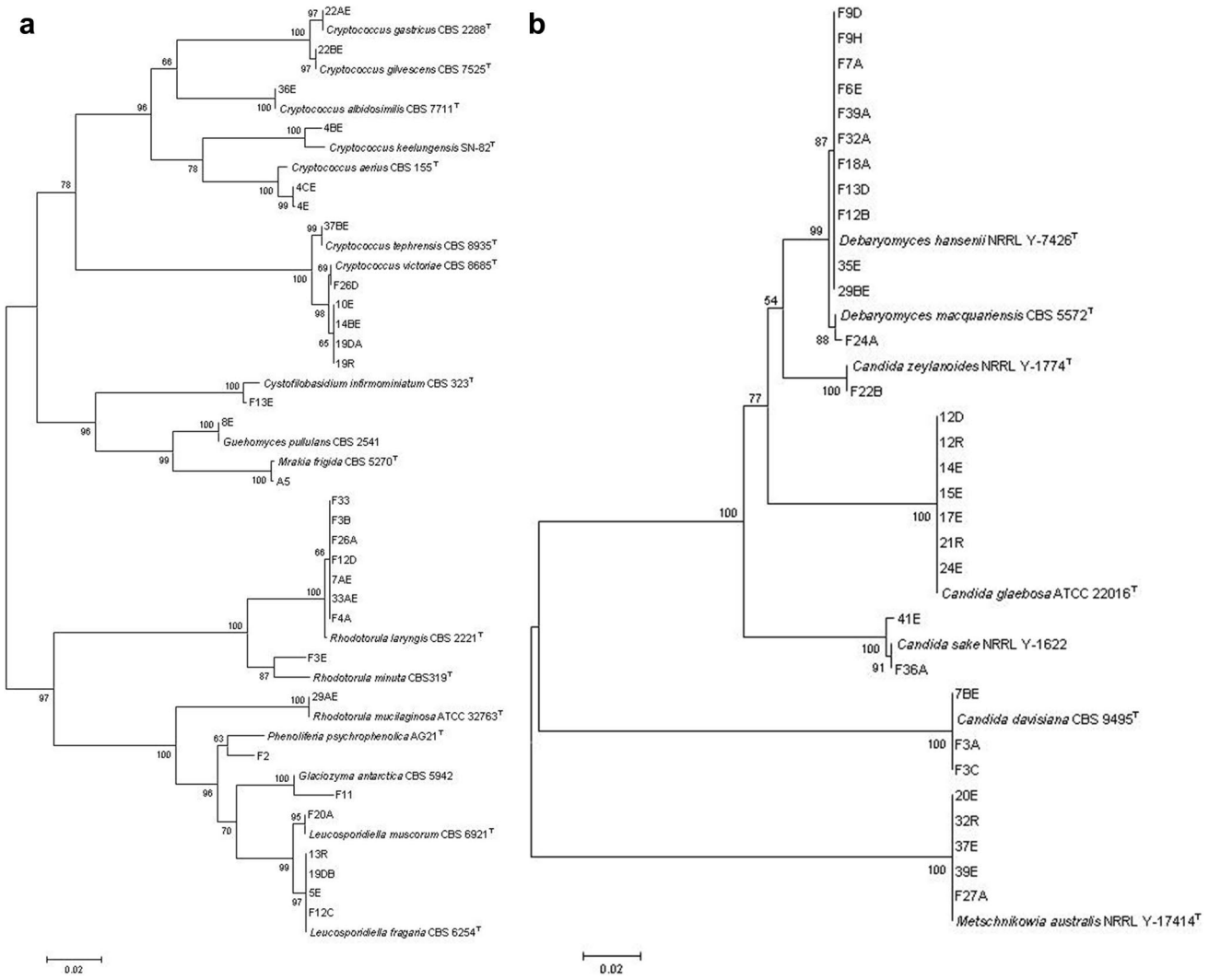


Fig. 2 **a** Phylogenetic tree based on the sequences of D1/D2 region from ascomycetous yeast isolates from King George Island, Antarctica. The tree was constructed using the Neighbor-Joining method. Bootstrap values (1000 tree interactions) are indicated at the nodes. **b** Phylogenetic tree based on the sequences of D1/D2 region from basidiomycetous yeast isolates from King George Island, Antarctica. The tree was constructed using the Neighbor-Joining method. Bootstrap values (1000 tree interactions) are indicated at the nodes

be well adapted to survive in unstable environments with fluctuating temperatures, such as Antarctica ice-free microhabitats. The culturable yeast community found in the present work showed moderate diversity and low dominance of species. In fact, sixty-one yeast isolates were distributed in 24 species, of which *Rhodotorula laryngis, Candida glaebosa* and *Debaryomyces hansenii* were the most widely represented. When the culturable yeast diversity obtained in this work was compared with that from other cold environments like European glaciers and Arctic environments, and also with previously reports of Antarctica, it can be seen a notable resemblance. In fact, most of the species, both psychrotrophic and psychrophilic, found in this work have been previously reported from different Antarctic and subantarctic zones. However, this is the first report on the occurrence of *Cryptococcus aerius* and *Cryptococcus keelungensis* in those environments.

Cryptococcus species have been frequently found in a variety of Antarctic sites and substrates, and many are psychrophilic (Vishniac 2006a). *Cryptococcus* species may be able to utilize available nutrients in oligotrophic systems and are heterogeneous in terms of their nutritional abilities (Connell et al. 2006).

Cryptococcus aerius, in particular, has been previously isolated from Arctic and European glaciers (Branda et al. 2010; Vishniac 2006a) and also from soils, sand and plant leaves from milder regions (Fonseca et al. 2000; Renker et al. 2004). On the other hand, *Cryptococcus keelungensis* was isolated for the first time by Chang et al. (2008) from a sea-surface microlayer at Keelung on the north-east coast

Table 2 Extracellular enzymatic activity of yeasts from King George Island

Yeast species	Number strains	Pr A		Es A (Tween 80)		Am A		In A		Pec A		Xil A	
		8 °C	20 °C	8 °C	20 °C	8 °C	20 °C	8 °C	20 °C	8 °C	20 °C	8 °C	20 °C
Candida glaebosa	7							1					
C. sake	2												
C. davisiana	3	3		2	3	1		1	1	2	1		
C. zeylanoides	1												
Cystofilobasidium infirmominiatum	1						1			1			
Cryptococcus aerius	2						1			1		1	
C. albidosimilis	1												
C. gastricus	1								1				
C. gilvescens	1					1	1						
C. tephrensis	1			1	1	1			1		1	1	1
C. victoriae	5	1		3	5			3	4	1	1		1
C. keelungensis	1	1	1	1	1	1						1	
Debaryomyces hansenii	11	1				1							1
D. macquariensis	1												
Guehomyces pullulans	1	1	1	1		1	1	1	1	1	1		1
Leucosporidiella fragaria	4	3	1	3	4				1			1	
L. muscorum	1		1	1									1
Metschnikowia australis	5	1		2	2	1		1	3	2	1	1	1
Rhodotorula laryngis	7	1		2	6								
R. minuta	1												
R. mucilaginosa	1												
Total no. of strains	58												
Total no. of positive strains		12	4	16	22	7	4	7	12	8	5	5	6
% of total strains positive		21	7	28	38	12	7	12	21	14	9	9	10

PrA proteolytic activity, *EsA* esterasic activity on Tween 80 agar, *AmA* amylolytic activity, *InA* inulolytic activity, *PecA* pectinolytic activity, *XilA* xilanolytic activity, *CelA* cellulolytic activity

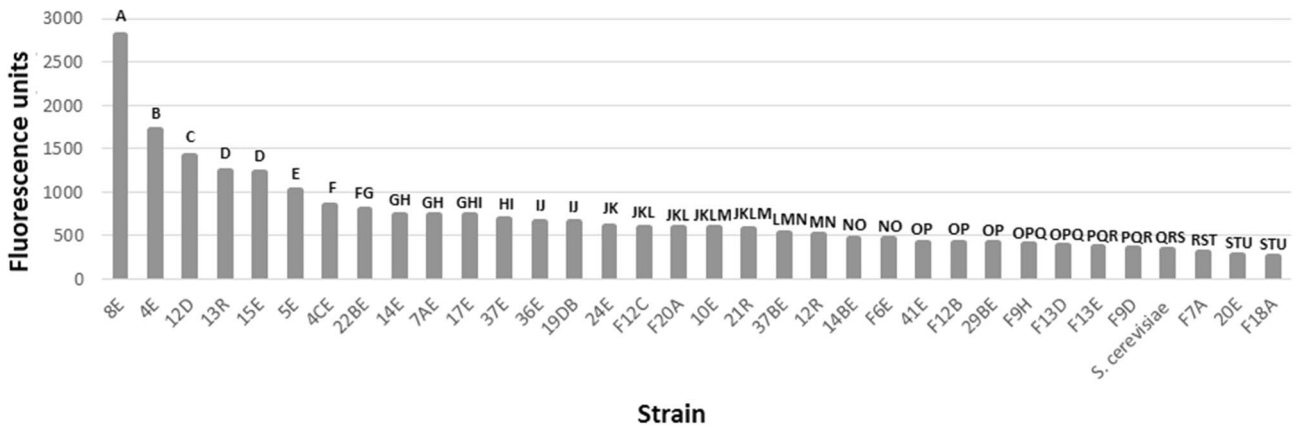


Fig. 3 Fluorescence of the yeast strain, the experimental data were analyzed for statistical significance using a one-way ANOVA, with comparison using the Fisher's LSD (least significant difference) test to determine the specific differences. Differences were considered significant at the level of $P < 0.05$. Significant differences as revealed by the Fisher LSD test are indicated by *different letters above the bars*

of Taiwan, and up to now all the reports of this species have been circumscribed to that location (Chang et al. 2016). Sea-surface microlayer is the interface of the sea and the atmosphere and it is considered an extreme environment due to its harsh conditions with high levels of visible and UV radiation and high concentration of pollutants. In this work the strain identified as *Cryptococcus keelungensis* was isolated from soil on the top of a hill close to Collins glacier. Since levels of UV radiation have increased worldwide but especially in Antarctica (Quesada and Vincent 1997) the presence of a yeast species with resistance to UV radiation was not surprising.

Only three of the yeast strains isolated in this work could be defined as obligate psychrophiles. They were identified as *Glaciozyma antarctica, Phenoliferia psychrophenolica* and *Mrakia frigida*. *Glaciozyma antarctica* and *Mrakia frigida* have already been isolated from Antarctic regions (Fell 2011; Turchetti et al. 2011), being this the first report of *Phenolipheria psychrophenolica*, as an Antarctic yeast. This species, previously named *Rhodotorula psychrophenolica*, is a true psychrophilic microorganism unable to grow at temperatures above 20 °C. It has been previously isolated from mud in the thawing zone of the Stubier glacier in Austria and the Etendard Glacier in France by Margesin et al. (2007). However, until now this species has not been recovered from Antarctica. It has been demonstrated that this species could degrade aromatic substrates like phenol, catechol, resorcinol, hydroquinone or benzoate (Margesin et al. 2007), so it could be of much help in bioremediation processes in cold environments.

Most of fungi found in Antarctica are cosmopolitan (Godinho et al. 2015) which is in accordance with our results. None of the yeast species found in this work are endemic of Antarctica, since they have been previously reported from other cold environments like Arctic region, European glaciers or even milder habitats. In this regard, Frisvad (2008) stated that most Antarctica fungal species have been introduced via humans, animals or wind dispersal and have developed adaptation strategies to survive in such cold and oligotrophic habitats (Vincent 2000). Thus, most of the yeast isolates studied in this work produced at least one of the tested extracellular enzymes when grown at low temperatures (8 °C).

Esterase activity was expressed by most of the yeasts in this work, consistent with a previous report from Carrasco et al. (2012). Inulinases were produced by 13 strains, mainly at 20 °C; however, in some cases activity was also expressed at 8 °C. This study constitutes the first report of the screening on inulinase activity carried out for yeast from Antarctica.

The least common extracellular enzymes were xylanase and amylase, observed only in 7 and 6 % of the isolates at 8 °C. Although cellulase activity has been previously described in cold-adapted yeast isolated from Antarctica (Carrasco et al. 2012), no cellulase activity was observed here. *Guehomyces pullulans* expressed all the tested enzymes, except cellulase. This species was previously isolated from Antarctic environments and studied for the capability to produce several enzymatic activities. Duarte et al. (2013) reported the production of proteases, xylanases and lipases by strains of this species. Moreover, the production of beta-galactosidases at 0 °C has also been documented by Nakagawa et al. (2006). However, the present work constitutes the first report of the production of extracellular amylases, inulinases, and xylanases by this species. *Guehomyces pullulans* has also been associated with transformation of some recalcitrant compounds which enhances its biotechnological potential (Sláviková et al. 2002).

It is worth to remind that all of the extracellular enzymatic activities analyzed in this work are of interest to industry: amylases and inulinases in food processing, fermentation and pharmaceutical industries. Inulinases have important industrial application in the production of pure fructose. Cellulases and pectinases are interesting in textiles, biofuel processing and clarification of fruit juices; esterases in agro-food industries and proteases in detergent formulation, food and beverage industries and environmental bioremediation. Finally, xylanases can be applied as hydrolysis agent in biofuel and solvent industries (Aurilia et al. 2008; Pulicherla et al. 2012).

Another characteristic of the studied yeasts that could be exploited is their capacity to accumulate intracellular lipids for the production of the "third-generation" biodiesel (Sitepu et al. 2014). About half of the yeast strains in this study accumulated more than 20 % of lipids by dry weight, and so they could be characterized as oleaginous microorganisms. Oleaginous yeasts have the capability to synthesize and accumulate high levels of triacylglycerides within their cells, up to 70 % of the biomass weight (Sitepu et al. 2013). It was not surprising to find oleaginous yeasts in Antarctica since many oleaginous species occupy relatively dry, oligotrophic habitats. It seems that storage lipids would presumably be more resilient to desiccating conditions than storage carbohydrates (Sitepu et al. 2014). *Guehomyces pullulans* 8E was the strain that accumulated the highest levels of intracellular lipids. As far as we know, strains of this species have not been characterized as oleaginous before. The strain presented here could accumulate 42 % of saponifiable lipids by dry weight. However, that value could be improved by the manipulation of culture conditions as demonstrated by Sitepu et al. (2013). They obtained higher lipid accumulation by a strain of *Rhodosporidium babjevae* from 39 to 46 % by dry weight, by changing the culture medium. Our results showed that the psychrotolerant strain 8E appears as a good candidate to be a source of lipids for biodiesel production. The ability to grow at a wide range of temperatures increases its potential

since when cultured at different temperatures, the fatty acid composition of its internal lipids would change, and so biodiesel with different characteristics could be obtained. In agreement with our results about growth temperatures, enzymes production and lipid accumulation, the biotechnological potential of *Guehomyces pullulans* 8E should be further explored.

Conclusion

In agreement with previous reports of microorganisms isolated from cold environments, the yeasts isolated in this work are predominantly psychrotolerant. The diversity of extracellular enzymes activities expressed by the yeasts isolated, and hence the diversity of compounds that they can degrade or transform, reflects the importance of the yeast community in nutrient recycling in the Antarctic regions. The Antarctic yeast isolates may potentially contribute to industrial processes that require high enzymatic activity at low temperatures, including bread, baking, textile, food, biofuel and brewing industries.

Acknowledgments This work was supported by grants from Agencia Nacional de Investigación e Innovación (ANII, FSE 102780), Instituto Antártico Uruguayo (IAU), Pedeciba, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, PIP 112-201101-00662) and Agencia Nacional de PromociónCientífica y Tecnológica (PICT 2014-1655).

References

Amaretti A, Raimondi S, Sala M, Roncaglia L, De Lucia M, Leonardi A, Rossi M (2010) Single cell oils of the cold-adapted oleaginous yeast *Rhodotorula glacialis* DBVPG 4785. Microb Cell Fact. doi:10.1186/1475-2859-9-73

Aurilia V, Parracino A, D'Auria S (2008) Microbial carbohydrate esterases in cold adapted environments. Gene 410:234–240. doi:10.1016/j.gene.2007.12.019

Branda E, Turchetti B, Diolaiuti G, Pecci M, Smiraglia C, Buzzini P (2010) Yeast and yeast-like diversity in the southernmost glacier of Europe (Calderone Glacier, Apennines, Italy). FEMS Microbiol Ecol 72:354–369

Buzzini P, Margesin R (2014) Cold-adapted yeasts: a lesson from the cold and a challenge for the XXI century. Coldadapt Yeasts Biodivers Adapt Strateg Biotechnol Signif 9783642396816:3–22. doi:10.1007/978-3-642-39681-6_1

Buzzini P, Martini A (2002) Extracellular enzymatic activity profiles in yeast and yeast-like strains isolated from tropical environments. J Appl Microbiol 93:1020–1025. doi:10.1046/j.1365-2672.2002.01783.x

Carrasco M, Rozas JM, Barahona S, Alcaíno J, Cifuentes V, Baeza M (2012) Diversity and extracellular enzymatic activities of yeasts isolated from King George Island, the sub-Antarctic region. BMC Microbiol. doi:10.1186/1471-2180-12-251

Chang CF, Lee CF, Liu SM (2008) *Cryptococcus keelungensis* sp. nov., an anamorphic basidiomycetous yeast isolated from the sea-surface microlayer of the north-east coast of Taiwan. Int J Syst Evol Microbiol 58:2973–2976. doi:10.1099/ijs.0.65773-0

Chang CF, Lee CF, Lin KY, Liu SM (2016) Diversity of yeasts associated with the sea surface microlayer and underlying water along the northern coast of Taiwan. Res Microbiol 167:35–45. doi:10.1016/j.resmic.2015.08.005

Connell L, Redman R, Craig S, Rodriguez R (2006) Distribution and abundance of fungi in the soils of Taylor Valley. Antarct Soil Biol Biochem 38:3083–3094. doi:10.1016/j.soilbio.2006.02.016

Connell LB, Redman R, Rodriguez R, Barrett A, Iszard M, Fonseca Á (2010) *Dioszegia antarctica* sp. nov. and *Dioszegia cryoxerica* sp. nov., psychrophilic basidiomycetous yeasts from polar desert soils in Antarctica. Int J Syst Evol Microbiol 60:1466–1472. doi:10.1099/ijs.0.015412-0

De García V, Brizzio S, Libkind D, Buzzini P, Van Broock M (2007) Biodiversity of cold-adapted yeasts from glacial meltwater rivers in Patagonia. Argent FEMS Microbiol Ecol 59:331–341. doi:10.1111/j.1574-6941.2006.00239.x

Di Menna ME (1966) Yeasts in Antarctic soils. Antonie van Leeuwenhoek 32:29–38

Di Rienzo JACF, Balzarini MG, Gonzalez L, Tablada M, Robledo CW (2009) InfoStat versión 2009. Universidad Nacional de Córdoba, Argentina

Duarte AWF et al (2013) Taxonomic assessment and enzymes production by yeasts isolated from marine and terrestrial Antarctic samples. Extremophiles 17:1023–1035. doi:10.1007/s00792-013-0584-y

Fell JW, Mrakia Y (2011) Yamada & Komagata (1987). Yeasts 3:1503–1510. doi:10.1016/b978-0-444-52149-1.00123-3

Fonseca A, Scorzetti G, Fell JW (2000) Diversity in the yeast *Cryptococcus albidus* and related species as revealed by ribosomal DNA sequence analysis. Can J Microbiol 46:7–27

Frisvad JC (2008) Fungi in cold ecosystems. In: Psychrophiles: from biodiversity to biotechnology, pp 137–156. doi:10.1007/978-3-540-74335-4_9

Garay LA et al (2016) Eighteen new oleaginous yeast species. J Ind Microbiol Biotechnol: 1–14

Godinho VM et al (2015) Diversity and bioprospection of fungal community present in oligotrophic soil of continental Antarctica. Extremophiles 19:585–596. doi:10.1007/s00792-015-0741-6

Hammer Ø, Harper DAT, Ryan PD (2001) Past: paleontological statistics software package for education and data analysis Palaeontol Electron 4:XIX–XX

Kimura K, Yamaoka M, Kamisaka Y (2004) Rapid estimation of lipids in oleaginous fungi and yeasts using Nile red fluorescence. J Microbiol Methods 56:331–338. doi:10.1016/j.mimet.2003.10.018

Margesin R, Miteva V (2011) Diversity and ecology of psychrophilic microorganisms. Res Microbiol 162:346–361. doi:10.1016/j.resmic.2010.12.004

Margesin R, Fonteyne PA, Schinner F, Sampaio JP (2007) *Rhodotorula psychrophila* sp. nov., *Rhodotorula psychrophenolica* sp. nov. and *Rhodotorula glacialis* sp. nov., novel psychrophilic basidiomycetous yeast species isolated from alpine environments. Int J Syst Evol Microbiol 57:2179–2184. doi:10.1099/ijs.0.65111-0

Mohamed Hatha AA, Rahiman Mujeeb RK, Krishnan KP, Saramma AV, Saritha G, Lal D (2013) Characterisation and bioprospecting of cold adapted yeast from water samples of Kongsfjord. Nor Arct Indian J Mar Sci 42:458–465

Morita RY (1975) Psychrophilic bacteria. Bacteriol Rev 39:144–167

Nakagawa T, Ikehata R, Uchino M, Miyaji T, Takano K, Tomizuka N (2006) Cold-active acid β-galactosidase activity of isolated psychrophilic-basidiomycetous yeast *Guehomyces pullulans*. Microbiol Res 161:75–79

Pereyra V, Martinez A, Rufo C, Vero S (2014) Oleaginous yeasts form Uruguay and Antarctica as renewable raw material for biodiesel production. Am J Biosci 2:251–257

Pulicherla K, Ghosh M, Kumar PS, Rao KS (2012) Psychrozymes—the next generation industrial enzymes. J Mar Sci Res Dev 2011

Quesada A, Vincent W (1997) Strategies of adaptation by Antarctic cyanobacteria to ultraviolet radiation. Eur J Phycol 32:335–342

Ratledge C, Hall MJ (1979) Accumulation of lipid by *Rhodotorula glutinis* in continuous culture. Biotechnol Lett 1:115–120. doi:10.1007/bf01386709

Renker C, Blanke V, Börstler B, Heinrichs J, Buscot F (2004) Diversity of Cryptococcus and Dioszegia yeasts (Basidiomycota) inhabiting arbuscular mycorrhizal roots or spores. FEMS Yeast Res 4:597–603

Rossi M et al (2009) Growth, lipid accumulation, and fatty acid composition in obligate psychrophilic, facultative psychrophilic, and mesophilic yeasts. FEMS Microbiol Ecol 69:363–372. doi:10.1111/j.1574-6941.2009.00727.x

Ruisi S, Barreca D, Selbmann L, Zucconi L, Onofri S (2007) Fungi in Antarctica. Rev Environ Sci Bio/Technol 6:127–141

Schena L, Ippolito A, Zahavi T, Cohen L, Nigro F, Droby S (1999) Genetic diversity and biocontrol activity of *Aureobasidium pullulans* isolates against postharvest rots. Postharvest Biol Technol 17:189–199

Shivaji S, Prasad G (2009) Antarctic yeasts: biodiversity and potential applications. In: Yeast biotechnology: diversity and applications. Springer, New York, pp 3–18

Sitepu IR et al (2012) An improved high-throughput Nile red fluorescence assay for estimating intracellular lipids in a variety of yeast species. J Microbiol Methods 91:321–328. doi:10.1016/j.mimet.2012.09.001

Sitepu IR et al (2013) Manipulation of culture conditions alters lipid content and fatty acid profiles of a wide variety of known and new oleaginous yeast species. Bioresour Technol 144:360–369. doi:10.1016/j.biortech.2013.06.047

Sitepu IR, Garay LA, Sestric R, Levin D, Block DE, German JB, Boundy-Mills KL (2014) Oleaginous yeasts for biodiesel: current and future trends in biology and production. Biotechnol Adv 32:1336–1360. doi:10.1016/j.biotechadv.2014.08.003

Sláviková E, Košíková B, Mikulášová M (2002) Biotransformation of waste lignin products by the soil-inhabiting yeast *Trichosporon pullulans*. Can J Microbiol 48:200–203

Thakur MS, Prapulla SG, Karanth NG (1989) Estimation of intracellular lipids by the measurement of absorbance of yeast cells stained with Sudan Black B. Enzyme Microb Technol 11:252–254

Tin T, Fleming ZL, Hugh KA, Ainley DG, Convey P, Morenos CA, Pfeiffer S, Scott J, Snape I (2009) Impacts of local human activities on the Antarctic environment. Antarc Sci 21:3–33

Turchetti B et al (2011) Psychrophilic yeasts from Antarctica and European glaciers: description of *Glaciozyma* gen. nov., *Glaciozyma martinii* sp. nov. and *Glaciozyma watsonii* sp. nov. Extremophiles 15:573–586. doi:10.1007/s00792-011-0388-x

Vincent WF (2000) Evolutionary origins of Antarctic microbiota: invasion, selection and endemism. Antarct Sci 12:374–385

Vishniac HS (2006a) A multivariate analysis of soil yeasts isolated from a latitudinal gradient. Microb Ecol 52:90–103. doi:10.1007/s00248-006-9066-4

Vishniac HS (2006) Yeast biodiversity in the Antarctic. In: Biodiversity and ecophysiology of yeasts. Springer, New York, pp 419–440

Vorapreeda T, Thammarongtham C, Cheevadhanarak S, Laoteng K (2012) Alternative routes of acetyl-CoA synthesis identified by comparative genomic analysis: involvement in the lipid production of oleaginous yeast and fungi. Microbiology 158:217–228

Zhang S, Skerker JM, Rutter CD, Maurer MJ, Arkin AP, Rao CV (2015) Engineering *Rhodosporidium toruloides* for increased lipid production. Biotechnol Bioeng