**Production of extracellular hydrolase enzymes by fungi from King George Island**

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**Abstract**

Fungi are known to produce a range of extracellular enzymes and other secondary metabolites. Investment in extracellular enzyme production may be an important element of the survival strategy of these fungi in maritime Antarctic soils. This study focuses on fungi that were isolated from ornithogenic, undisturbed and human-impacted soils collected from the Fildes Peninsula, King George Island, Antarctica during the austral summer in February 2007. We (i) describe fungal diversity based on molecular approaches, (ii) describe the thermal characteristics of the fungal isolates, and (iii) screen extracellular hydrolase enzyme production (amylase and cellulase) by the isolates. Soil samples were cultured using the Warcup soil plating technique and incubated at 4ºC and 25ºC to allow basic thermal classification. In total, 101 isolates were obtained. All the isolates were screened at culture temperatures of 4ºC and 25ºC in order to detect activity of extracellular hydrolase enzymes. At 25ºC, ornithogenic penguin rookery soils recorded the lowest diversity of fungi, with little difference in diversity apparent between the other soils examined. At 4ºC an undisturbed site recorded the lowest and a human-impacted site the highest diversity of fungi. The majority of the fungi identified in this study were in the mesophilic thermal class. Six strains possessed significant activity for amylase and 13 for cellulase at 25ºC. At 4ºC, four strains showed significant amylase and 22 significant cellulase activity. The data presented increase our understanding of microbial responses to environmental temperature.

Keywords: Extracellular enzymes, secondary metabolites, psychrophilic, psychrotolerant, mesophilic, screening

**Introduction**

Antarctic soils are typically poorly developed, with low nutrient content compared with soils in other parts of the world (Beyer and Bölter 2000). This contributes to the limited vegetation development in this region. External nutrient sources such as nitrogen deposited by penguins and other vertebrates therefore have an important role in Antarctic terrestrial ecosystems (Greenfield 1992; Bokhorst et al. 2007). Abandoned penguin colonies also have rich ‘legacy’ deposits of guano, which can serve as a nutrient source for flora and microbiota (Tatur et al. 1997).

Antarctica provides an extreme and harsh environment for terrestrial life (Block 1984). Abundance and diversity of organisms decreases with altitude and latitude from the coast to the continental plateau (Pickard and Seppelt 1984; Kappen 1993; Broady 1996). Biodiversity in Antarctica is greater in the coastal areas both in the maritime and continental Antarctic (Convey et al. 2014). This cold environment is dominated by a range of microorganisms such as archaea, bacteria, fungi, actinomycetes and algae (Margesin and Miteva 2011). The macroscopic organisms present are soil invertebrates, including Diptera, Acari, Collembola, Nematoda, Rotifera, Tardigrada, and Protista (Block 1984; Adams et al. 2006; Convey 2013) and lower plants including mosses, liverworts and lichens (Smith 1984; Convey 2013). Only two vascular plants are present in Antarctica, *Deschampsia antarctica* Desv. and *Colobanthus quitensis* (Kunth) Bartl.

Fungi are a dominant microorganism group in the soils of Antarctica (Yergeau et al. 2007; Onofri et al. 2007a). They are functionally important in soil ecosystems, being partly responsible for the decomposition and release of dead organic matter and plant nutrients (Read and Perez-Moreno 2003). They also participate in mycorrhizal associations (Upson et al. 2008). Mycorrhiza-like associations have also been reported with liverworts as far south as Granite Harbor (Victoria Land) (Williams et al. 1994; Newsham 2010). Soil fungi are also increasingly being assessed in bioprospecting studies, for instance in the production of antibiotics (O’Brien et al. 2004; Wong et al. 2011).

Fungal studies conducted in the polar regions to date have focussed on morphology (Del Frate and Caretta1990; Montemartini Corte 1991; Onofri et al. 1991, 1999; Onofri and Tosi 1992; Mercantini et al. 1993; Montemartini Corte et al. 1993), ecophysiology (Caretta et al. 1994; Zucconi et al. 1996, 2002; Fenice et al. 1997; Onofri et al. 2000; Tosi et al. 2002, 2004; Selbmann et al. 2011), ecology (Zucconi et al. 2002; Selbmann et al. 2012, 2013), molecular biology (Vishniac and Onofri 2002; Selbmann et al. 2005; Onofri et al. 2007b), phylogeny (Upson et al. 2007; Newsham and Bridge 2010; Egidi et al. 2014), bioactivity (Nichols et al. 2002; Brunati, et al. 2009) and enzymology (Duncan et al. 2006; Krishnan et al. 2011).

The production of extracellular metabolites is a widespread feature of microbial biology, these compounds contributing to diverse functions including resource acquisition, protection, competition, and inter- and intraspecific interactions. Fungi are known to produce a range of extracellular enzymes, in particular hydrolases, which will aid in their acquisition of nutrients from the surrounding environment. A small number of studies have addressed this aspect of their biology in Antarctica (Fenice et al. 1997; Bradner et al. 1999; Kasieczka-Burnecka et al. 2007; Krishnan et al. 2011), with the majority of the reports being from the continental Antarctic (Margesin et al. 2007). However, most enzyme studies from the Antarctic region have focused on bacteria and in some cases on fungi (yeasts) (Birgisson et al. 2003; Gesheva and Vasileva-Tonkova 2012; Carrasco et al. 2012).

Extracellular hydrolase enzymes (EHEs) generally function to degrade soil organic matter (SOM) with the products subsequently being absorbed by the producing cells. In addition to SOM deriving from primary production, it has also been reported that soil microfungi contribute significantly in the bioremediation of hydrocarbon-contaminated soil in Antarctica (Ferrari et al. 2011). In the current study we focused on amylase and cellulase activity, as these enzymes have been widely reported in fungal studies elsewhere globally but not from Antarctica (Tortella et al. 2008; Brindha et al. 2011; Vega et al. 2012).

This study set out to i) improve knowledge of the diversity of soil microfungi from King George Island, Antarctica, through the application of molecular methods; ii) describe the thermal growth characteristics of the fungal isolates obtained and iii) screen for extracellular hydrolase enzyme production. The study develops that of Krishnan et al. (2011) through examining isolates obtained from different sampling locations and in the identification and screening of fungal isolates and their activity from all three thermal classes of mesophilic, psychrotolerant and psychrophilic fungi.

**Materials and Methods**

*Soil sampling and fungal isolation*

Soil samples were collected during the austral summer in February 2007 at King George Island, South Shetland islands, from an undisturbed vegetated area (62º10’24.5”S, 58º56’45.3”W), a penguin rookery (62º12’34.7”S, 58º55’33.0”W), an area of ornithogenically influenced vegetation (62º11'37"S, 58º59'35"W) and a human impacted site (62º12’00.7”S, 58º57’35.6”W) (Figure1a,b,c). Soil fungi were cultured on Potato Dextrose Agar (PDA) media using Warcup’s (1950) soil plating method. Approximately 0.1g soil was placed in sterile Petri dishes into which sterilized PDA medium supplemented with chloramphenicol (0.2 g/l) was poured. Replicate plates (n = 6) for each sampling site were prepared, with three incubated at each of 4°C and 25°C, to permit a pragmatic thermal classification into mesophilic, psychrophilic and psychrotolerant strains. Isolates growing at 4°C only were classified as psychrophilic, those at 25°C only as mesophilic and those at both temperatures as psychrotolerant. To obtain individual isolates, the visible active growing mycelia were then taken from the mixed isolate and subcultured onto PDA. Isolates obtained at 4ºC and 25ºC continued to be incubated at these temperatures. After maturation in culture, fungi were identified using molecular methods. Fungal strains were deposited in the Institute of Biological Science, University Malaya, fungal collection which is located in the National Antarctic Research Center, Kuala Lumpur.

*Molecular identification of selected fungi*

The fungal isolates were grown in PDA media. The DNeasy Plant DNA Extraction Kit (Qiagen) was used to extract genomic DNA according to the manufacturer’s instructions. The intergenic spacer regions of the nuclear rRNA genes were amplified using primer pairs ITS4/ITS5 (White et al. 1990). PCR reactions were performed in a 50 µl volume containing ca. 20 ng DNA, 0.2 µM concentration of each primer, 0.2 mM concentrations of each dNTP, 2.5 mM MgCl2 and 1.25 U of Taq Polymerase (Invitrogen). The amplification cycle involved an initial denaturation step of 95°C for 2 min followed by 35 cycles of denaturation (95°C for 1 min), annealing (54°C for 1 min) and elongation (72°C for 1.5 min). A final 10-min elongation step at 72°C was used. Agarose gel electrophoresis was used to analyse PCR products, which were then sent to Tri-I Biotech. Inc., Taiwan, for sequencing. The sequences obtained were checked for ambiguity, assembled and submitted to the National Center for Biotechnology Information (NCBI) for a nucleotide BLAST search. The nearest neighbor sequence identities from the database was taken as the identity of the fungal isolate.

*Enzyme screening*

Extracellular hydrolase enzyme activity was screened as described by Margesin et al. (2003). The presence of amylase and cellulase activity were tested, respectively, on R2A agar (casein acid hydrolysate 0.5g/l, yeast extract 0.5g/l, proteose peptone 0.5g/l, dextrose 0.5g/l, soluble starch 0.5g/l, dipotassium phosphate 0.3g/l, magnesium sulphate 0.024g/l, sodium pyruvate 0.3g/l, agar 15g/l) supplemented with either starch (Cat number: S9765 Sigma Aldrich) (0.4% w/v) or carboxymethylcellulose (Cat number: 419338 Sigma Aldrich) and trypan blue (Cat number: 76146 Sigma Aldrich) (0.4% and 0.01% w/v) respectively.

All test fungi were subjected to the agar plug assay method and prepared in three replicates. Agar plugs (6 mm) were bored from the edge of the fungal colonies using cork borer number 3 and inoculated into a well of the same size made at the centre of each assay agar plate. Plates were then incubated at 4°C. After 10 d, the plates were examined for the presence of a clear zone in the agar around the colony, indicating extracellular enzyme activity. *Antarctomyces psychrotrophicus* was used as a control since it has no activity for any of the enzymes screened. Amylase activities were confirmed by staining the plates with Lugol’s solution.

*Relative enzyme activity (RA)*

Fresh samples were used for enzyme assays whenever possible in order to ensure that the enzyme activity was maximal (German et al. 2011). Each replicate was examined for the presence of a clear zone around the colony, and the diameter of the colony and of the clear zone (activity zone) were measured. The measurement was repeated in two mutually orthogonal dimensions, and the mean value calculated. The ‘relative enzyme activity’ (RA) was calculated using the following formula:



Isolates exhibiting an RA of >1.0 were classified as having ‘significant activity’ (Bradner et al. 1999; Duncan et al. 2008).

**Results**

*Identification and thermal classification of fungi*

From total of 49 fungal isolates obtained at 4°C, 43 were identified through BLAST search (Table 1a). Of these, only two belonged to Zygomycota, with the remainder being Ascomycota. Fifty-three fungal isolates were obtained at 25°C, of which 24 taxa were identified in a BLAST search (Table 1b). These included two Basidiomycota, 14 Ascomycota, five showing closest similarity to ‘uncultured fungus’, two with closest similarity to ‘Fungal Sp.AB34’ and a fungal endophyte. Thus, about 82% of the isolates obtained belonged to Ascomycota. Of the 21 distinct species identified, 15 were mesophiles, five were psychrophiles and one psychrotolerant (Figure 2a).

 The most common genus cultured at 4°C was *Geomyces*, while *Sporothrix* was most frequent at 25°C. Representatives of *Geomyces* were isolated from all human impacted, pristine and ornithogenic sites. In contrast, *Sporothrix* was only obtained from the human impacted site (Figure 2b,c).

*Enzyme screening*

Forty-two isolates were screened for enzyme activities at 4°C, including both identified and unidentified isolates. Thirty isolates showed activity for amylase but, of these, only four showed significant activity. The latter included three isolates of *Geomyces* sp. and an unidentified isolate. Cellulase screening revealed 38 isolates showing positive activity, of which 22 were significant producers. These included 20 *Geomyces* and two *Pseudeurotium* isolates. Figure 3a shows relative enzyme activities at 4°C for selected fungal isolates.

Thirty-one isolates were screened for enzyme activities at 25°C. Of these, 27 showed amylase activity. However, only six showed significant activity, including three *Geomyces*, one *Pseudeurotium*, one *Phialemonium* and one unidentified isolate. Twenty-eight isolates showed positive activity for cellulase, of which 13 showed significant activity. These included four isolates of *Geomyces*, two Fungal sp. AB34, one fungal endophyte, one *Galerina fallax*, one *Glomerella* and four unidentified fungi. The relative enzyme activities of selected isolates at 25°C are shown in Figure 3b.

**Discussion**

The highest fungal diversity at both culture temperatures was obtained from the human impacted site, suggestive of a relationship with human activity. Several studies have emphasized the potential role of humans in distributing microbiota to and within Antarctica (e.g. Azmi and Seppelt, 1997, 1998; Chown and Gaston 2000; Duncan et al. 2006; Krishnan et al. 2011). Indeed, Cowan et al. (2011) note that the human body is capable of supporting populations of more than 1012 microorganisms. However, to place such studies in context, it has also been reported that Whalers Bay (Deception Island), which is one of the most visited locations in Antarctica, has reduced microbial diversity in comparison with sites around research stations (Chown et al. 2005).

*Geomyces* spp.were frequently isolated at both 4°C and 25°C. *Geomyces* has previously been reported widely throughout Antarctica (Onofri et al. 2007a; Arenz and Blanchette 2011; Strauss et al. 2012; Alias et al. 2013), and was isolated at high frequency from all the current sampling sites at 4°C. Previously, this taxon has been reported from pristine sites (Kerry, 1990) and sites with very limited animal impacts (Azmi and Seppelt, 1997), as also noted here. Several studies have reported *Geomyces* to be a dominant genus in Antarctica (Tosi et al. 2002; Farrell et al. 2011). In the present study, strains of *Geomyces* were the only taxa obtained from ornithogenic sites, suggesting they may play a vital role in degrading keratin from feathers in these low temperature environments (Del Frate and Caretta, 1990; Marshall, 1998). Similar observations have been reported from Hornsund on High Arctic Svalbard, where sites associated with ornithogenic activity hosted *Geomyces pannorum* (Ali et al. 2013).

The most frequently encountered fungal class in this study was the Ascomycota (anamorphic ascomycetes), as is also noted globally (Blackwell 2011). Anamorphic ascomycetes tend to have short life cycles and limit their metabolic investment in sexual reproduction (Ruisi et al. 2007). It has been reported that anamorphic fungi are assisted in colonising some extreme environments through their ability to tolerate toxic substrata, unlike other groups of fungi (Thomas and Hill 1976; Jongmans et al. 1997). Studies from Ross Island (Arenz et al. 2011; Alias et al. 2013; Farrell et al. 2011), Macquarie Island (Ferrari et al. 2011), and an integration of data published across the entire Antarctic region (Bridge and Spooner 2012), have reported the dominance of this group. However, exceptionally, other groups have been reported to be dominant in specific studies in Antarctica, such as that of Connell et al. (2008), who described a community comprising 89% basidiomycetous yeasts and only two species of ascomycetes from Victoria Land.

Two species of Basidiomycota were identified from King George Island at a culture temperature of 25°C, *G. fallax* and *Schizophyllum commune.* The genus *Galerina* has previously been reported from maritime and sub-Antarctic regions (Pegler et al. 1980), including the Danco Coast (Gamundi and Spinedi 1988), Kerguelen Island (Pegler et al. 1980), South Georgia (Smith 1994), the South Sandwich Islands (Convey et al. 2000) and King George Island (Guminska et al. 1994). This genus has also been reported from Arctic tundra (Miller 2002). However, to our knowledge, neither *G. fallax* nor the genus *Shizophyllum* have previously been reported from Antarctica (Bridge and Spooner 2012).

In the present study, the majority of fungi isolated were mesophilic rather than psychrophilic or psychrotolerant, consistent with the observations of Azmi and Seppelt (1997) and Duncan et al. (2006). Moller and Dreyfuss (1996), also working on King George Island, reported a majority of fungal isolates to be psychrotolerant, although in reality this represented only 2% more than mesophilic isolates. The occurrence of mesophilic and psychrotolerant fungi may be indicative of fungal adaptation in the fluctuating temperatures typical of the maritime Antarctic terrestrial environment (Peck et al. 2006; Selbmann et al. 2012).

Cold-active enzyme studies are receiving increasing attention through their importance in biotechnology, especially in the context of energy and cost savings (Quanfu et al. 2012; Duarte et al. 2013). In the present study, several isolates were identified to possess significant amylase or cellulase activity. Production of these extracellular hydrolase enzymes by various microorganisms has also been described from Wilkes Land (Gesheva and Vasileva-Tonkova 2012), King George Island (Carrasco et al. 2012; Loperena et al. 2012) and the Larsemann Hills (Singh et al. 2013).

Cellulase showed different production patterns at the two different incubation temperatures used in the current study. At 4°C, 94% of the 49 strains isolated were able to produce cellulase, of which 53% were significant producers. Strains of *Geomyces* demonstrated significant cellulase production at 4°C. At 25°C, 93% of strains isolated were able to produce cellulase, with 43% being significant producers. Previous reports from King George Island have also demonstrated cellulase production by *Mrakia frigida* (Krishnan et al. 2011; Carrasco et al. 2012), a species not isolated here. In the present study, two isolates of *Geomyces* sp., *G. fallax*, a fungal endophyte and two isolates of *Pseudeurotium* sp. were identified to be the top three significant cellulase producers at the two culture temperatures. Enzyme activity at 25°C tended to be greater than that at 4°C, as evidenced by RA > 2.0, However, a greater number of strains isolated at 4°C than at 25°C were significant cellulase producers. The largest number of isolates capable of significant cellulase production were obtained from the human impacted sampling site, which may relate to this site being enriched in organic matter (cf. Duncan et al. 2006; Krishnan et al. 2011).

About 72% of isolates produced amylase but only 7.7% showed significant activity at 4°C, while the proportions were 92.9% and 21.4%, respectively, at 25°C. A similar study conducted on King George Island reported very little amylase activity in 10 fungal isolates (Carrasco et al. 2012). *Geomyces* isolates were again the primary group exhibiting significant amylase activity. All *Geomyces* isolates showed positive results in amylase screening, with the strongest activity being showed by those isolated from the human impacted site.

The fungal communities tested for enzyme activity here generally showed positive and significant results, suggesting the soil microfungal community plays an important role in decomposition processes in the Antarctic. Duncan et al. (2008) considered isolates with RA ≥ 1.0 as significant producers, whilst several of the isolates listed here had RA > 2.0. Therefore, we conclude that *Geomyces* sp., *Glomerella* sp., *Pseudeurotium* sp. and *G. fallax* merit further study. Further investigations should include determination of optimum culture conditions, purification and enzyme kinetic studies.

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