1.0 Introduction

1.1 Antarctic terrestrial environment

Antarctica is the driest and coldest place known on the Earth but the climate is not uniform throughout the continent (Øvstedal and Smith, 2001). In the winter, the coldest temperature is typically in the range of -20ºC to -50ºC, and much lower temperatures have been recorded. During the Antarctic summer, some parts of the continent never reach above -15ºC (or even lower). However temperature can be much higher, depending on the geographical position and climate at any specific location (Nienow and Friedmann, 1993). The lowest temperature ever recorded on Earth (-89.2°C) was recorded from the Russion Vostok Station in continental Antarctica (Ainsley *et al*., 2009). The continent's area covers about 10% of the Earth's land surface (Ainsley *et al*., 2009). Even though Antarctica is known as the 'white continent', ice-free land in Antarctica does exist, representing 0.34% of its total area (Fox and Cooper, 1994; Kanda and Komarkova, 1997), with the remainder being covered by ice sheets and permanent snow.

Antarctica can be divided into three terrestrial biogeographical zones based on the climate and biological characteristics (Onofri, Zucconi and Tosi, 2007; Convey, 2007). These are the sub-Antarctic, maritime Antarctic and continental Antarctic (Figure 1.1). The sub-Antarctic region comprises South Georgia, Marion Island, Prince Edward Island, Heard Island, Kerguelen Island, Crozet Island, McDonald Island and Macquarie Island. A cool oceanic climate, and mean monthly air temperature above 0°C for at least 6 months are two defining characteristics of this region. The maritime Antarctic includes the South Shetland Islands, South Orkney Islands, South Sandwich Islands, the Palmer Archipelago and the

north-east and west coasts of the Antarctic Peninsula, areas lying between 55 and 72°S (Onofri *et al*., 2007b; Ludley and Robinson, 2008). This region has a milder climate compared to continental Antarctica and the available water and vegetation development are greater (Ludley and Robinson, 2008). The continental Antarctic includes the main body of the continent and also the eastern coast of the Antarctic Peninsula (Onofri *et al*., 2007b; Convey, 2007), and has the harshest climate of the three zones. This region has a cold arid climate even in coastal areas (Longton, 1988; Onofri *et al*., 2007b).

Antarctic soils are typically poorly developed with low nutrient content compared with soils in other parts of the world (Beyer and Bölter, 2000). This may explain 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). Unoccuppied penguin colonies have rich deposits of guano, which can serve as a nutrient source for flora and microbiota (Tatur, Myrcha and Niegodzisz, 1997).

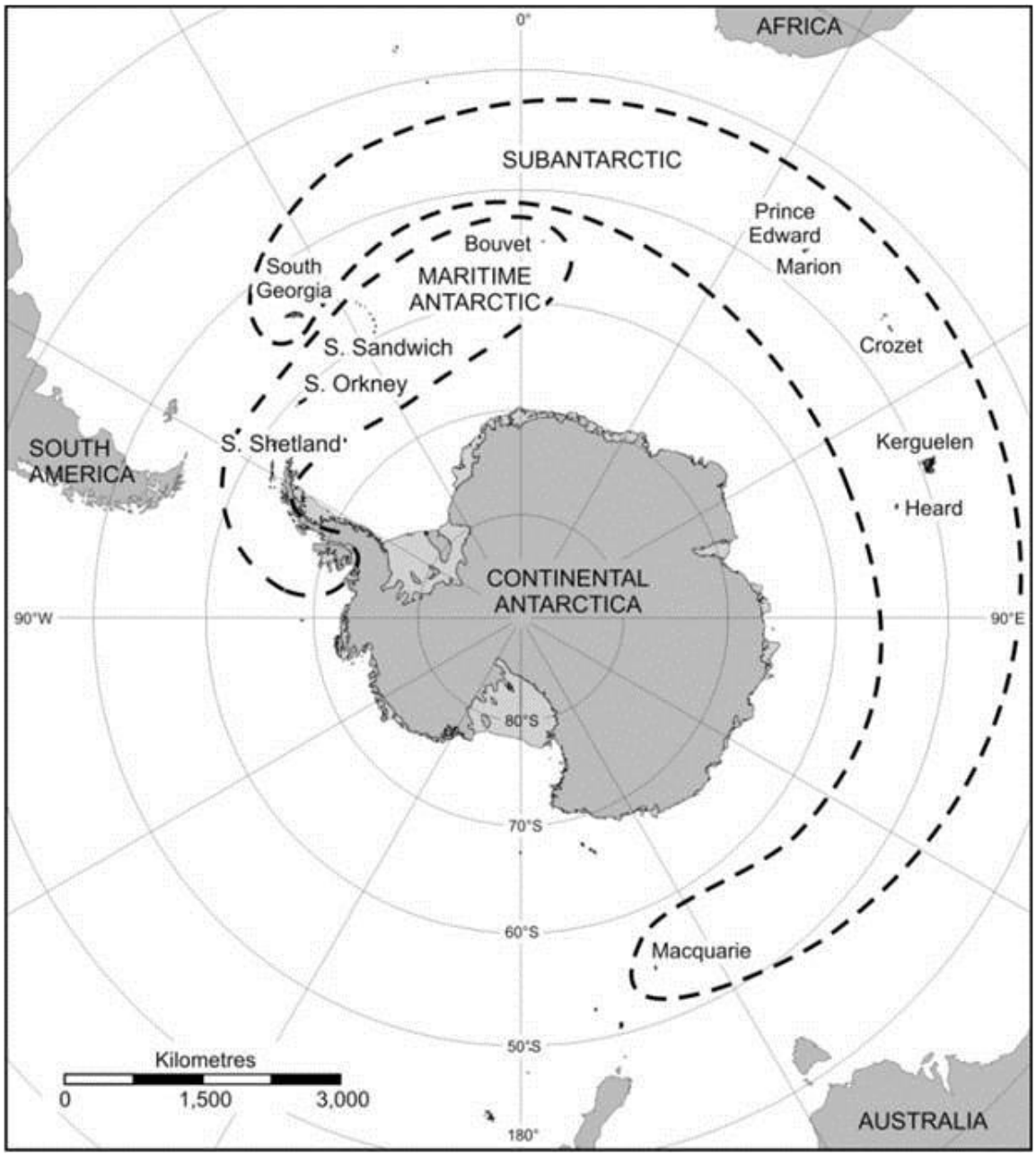


Figure 1.1: Terrestrial biogeographical zones of Antarctica

1.2 Geological history of Antarctica

The Antarctic Peninsula and South America were once contiguous (Clarke *et al*., 2005). These two regions were separated by the opening of the Drake Passage during the Cenozoic era, around 30 million years ago (Lawver and Gahagan, 2003). The final disintegration of Gondwanaland separated Australia and South America from Antarctica about 30 million years ago, subsequently allowing the formation of the Antarctic Circumpolar Current (Clarke *et al*., 2005; Bargagli, 2008) isolating the continent and Southern Ocean, and encouraging the cooling that led to the formation of continent-wide ice sheets. Therefore it is believed the current isolated position of Antarctica was reached about 34 million years ago (Abram *et al*., 2009). A large variety of plant and animal fossils found around the northern Antarctic Peninsula and dating from the late Cretaceous and early Cenozoic eras (Francis *et al*., 2008) provide support for this history.

1.3 Terrestrial biodiversity of Antarctica

Antarctica provides an extreme and harsh environment for terrestrial life (Block, 1984). Abundance and diversity of organisms decreases when altitude and latitude increase from the coast to the ice slope region (Pickard and Seppelt, 1984; Kappen, 1993; Broady, 1996). Biodiversity in Antarctica is greater in the coastal areas both in Peninsula and east Antarctica (Convey, 2010). This cold environment is dominated by a range of microorganisms such as yeast, 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, 2007) and lower plants including mosses, liverworts and lichens (Smith, 1984; Convey, 2007). Only two vascular plants are present in Antarctica, *Deschampsia antarctica* Desv. and *Colobanthus quitensis* (Kunth) Bartl.

King George Island is the largest Island in South Shetland Island archipelago. In the absence of published soil microclimatic data from the archipelago, studies on Signy Island from South Orkney Islands (which has a similar climate to the South Shetland Island group) show that minimum soil temperatures during winter remain at -5°C to -9°C, through insulation from overlying snow. However, short-term air temperatures can be much lower. During the austral summer, the soil habitat typically experiences low positive mean temperatures, although the absolute range of variation can be high (Peck, Convey and Barnes, 2006).

Fildes Peninsula is an ice-free area on King George Island which is one of the most visited and highly populated areas of the island, hosting several research stations (Braun *et al*., 2001). The terrestrial ecosystems of the Fildes Peninsula are varied, including rock and boulder field, pristine soil and vegetation, penguin rookeries, seal haul-out areas and human impacted sites (Smith, 1984; Tin *et al*., 2009). Terrestrial biodiversity comprises mosses, lichens, bacteria, microfungi, algae, microarthropods and microinvertebrates, seals, penguins, and marine birds such as skuas, gulls and petrels. Five different national research stations are located on the Fildes Peninsula, those of Chile, Uruguay, Russia, Korea and China. Both increasing numbers of tourists and the scientific activity in this congested area have affected the ecosystems of the Fildes Peninsula (Braun *et al*., 2001).

1.4 Microbial research in Antarctica

Since the beginning of the 20th Century microbiologists have shown interest in Antarctica (Bommer and Rousseau, 1905). Flint and Stout (1960) cultured fungal mycelia from the McMurdo Sound region, and Tubaki (1961) reported fungal species from the same area and from near Syowa Station. Studies such as these initiated microbial research in Antarctica.

Much research has been conducted on psychrophilic bacteria and archaea (e.g. Voytek *et al*., 1999; Delille and Delille, 2000; Chattopadhyay and Jagannadham, 2001; Baranecki *et al*., 2002; Cavicchioli *et al*., 2002; Deming, 2002; Yoshimune *et al*., 2005) and algae (e.g. Teoh *et al*., 2004; Irving *et al*., 2005; Sabacka and Elster, 2006; Wong *et al*., 2007), as well as a number of studies on fungi (Abyzov, 1993; Tosi *et al*., 2001; Gunde-Cimerman *et al*., 2003; Tosi *et al*., 2005; Ruisi *et al*., 2007; Alias *et al*., 2006). These studies have included molecular, phylogenetic, physiological and biotechnological approaches (Onofri *et al*., 2007a). Kanda and Komarkova (1997) suggested that terrestrial microbial diversity in Antarctica is lower than at corresponding latitudes in the northern hemisphere. However, there are recent suggestions that microbial diversity in Antarctica is much greater than was thought previously (Cowan *et al*., 2002; Vyverman *et al*., 2010). Generally microbiological studies in Antarctica have focused on the sub-Antarctic islands, Antarctic Peninsula and McMurdo Dry Valleys region of Southern Victoria Land (Vischniac, 1993; Arenz *et al*., 2006; Connell *et al*., 2006).

1.5 Thermal classification of microbes

The definition of thermal classes in microbiological studies varies between authors. Micro-organisms which are adapted to cold and able grow at low temperatures near 0°C were defined as psychrophiles or psychrotolerant by Gounot (1991). However Morita (1975) described psychrophiles as having optimum growth at 15°C or lower and being unable to grow at 20°C and above, while psychrotolerant organisms have their maximum temperature for growth above 20°C. Previously the latter were known as facultative psychrophiles. Both psychrophilic and psychrotolerant organisms can survive at low temperatures, and include deep sea organisms (-1ºC to 4ºC), Arctic and Antarctic organisms (-1ºC to -35ºC during winter) and glacial ice habitats (-5ºC) (Feller and Gerday, 2003). According to Cavicchioli *et al*. (2002), psychrophilic organisms grow fastest at 15°C and are not able to grow above 20°C. This statement is consistent with the definition of Margesin, Neuner and Storey (2007). *Cryptococcus vishniacii* Vishniac and Hempfling 1979, which is known to grow at -3°C (Vishniac and Hempfling, 1979), and *Mrakia frigida* (Fell, Statzell, Hunter & Phaff) Y. Yamada & Komag. 1987, which can grow below -7°C, are examples of Antarctic psychrophiles (Larkin and Stokes, 1968).

Psychrophilic and psychrotolerant microorganisms can play an important role in biotechnology, enzymology, the food industry and medicine (Margesin and Schinner, 1994). They possess adaptations to cold environments through physiological and ecological mechanisms (Robinson, 2001). In addition to psychrophilic and psychrotolerant microbes, a range of thermophilic and mesophilic microbes are known to exist in the Antarctic region. The former are recorded in geothermally heated soils (Gushterova *et al*., 2005; Convey and Smith, 2006; Vazquez, Coria and Mac Cormack, 2008). Thermophilic microbes have a

minimum temperature for growth of 20°C and maximum of at least 50°C (Magan, 2007). Their optimum temperature is in the range 40-50°C. Mesophilic microbes persist at intermediate temperatures. This group has a minimum temperature for growth between 5 and 10°C and maximum temperature above 25°C (Robinson, 2001).

1.6 Microfungi in Antarctica

Sclerotium antarcticum E. Bommer & M. Rousseau 1900 from Danco Coast, Antarctica was first fungi reported by Bommer and Rousseau (1905). Since then, more than 1000 fungi have been reported from the sub-, maritime and continental Antarctic (Ludley and Robinson, 2008; Bridge, Spooner and Roberts, 2009). The vast majority, 99.4%, of fungi reported from Antarctica are true fungi, comprised of filamentous fungi and yeasts (Onofri *et al*., 2007a). The majority group of fungi that has been reported from the Antarctic are hyphomycetes (Tubaki, 1961; Sun, Huppert and Cameron, 1978). There are 425 species currently reported from maritime Antarctica, of which 77 are recorded from King George Island (Bridge *et al*., 2009). Generally fungal data from the South Shetland Islands are limited to reports from Deception Island, King George Island and Elephant Island (Singer, 1957; Cameron and Benoit, 1970; Pegler, Spooner and Smith, 1980; Riemann and Shrage, 1983). Fungal studies conducted in Antarctica are focussed morphology (Del Frate and Caretta, 1990; Montemartini Corte, 1991; Onofri *et al*., 1991; Onofri and Tosi, 1992; Mercantini *et. al*., 1993; Montemartini Corte, Caretta and Del Frate, 1993), ecophysiology (Caretta, Del Frate and Mangiarotti, 1994; Zucconi *et al*., 1996; Fenice *et al*., 1997; Onofri *et al*., 2000; Tosi *et al*., 2002), molecular biology (Vishniac and Onofri, 2002) and

phylogeny (Onofri *et al*., 2007b; Upson, Read and Newsham, 2007; Newsham and Bridge, 2010).

The fungal species found in Antarctica can be divided into indigenous species and non-indigenous species (Vishniac, 1996; Ruisi *et al*., 2007). However it is difficult to determine which is indigenous and which is not (Tosi *et al*., 2002; Ruisi *et al*., 2007). Non-indigenous species are most probably brought to Antarctica from South America by humans (Ruisi *et al*., 2007). Fungi that can grow actively and reproduce in Antarctica are referred to as true indigenous species (Ruisi *et al*., 2007). Blanchette *et al*. (2010) reported that most of the fungi isolated from their study were indigenous to Antarctica. The presence of both non-indigenous and indigenous species was reported by Connell *et al*. (2006), who found *Penicillium* sp., a cosmopolitan species, and an unidentified black yeast throughout the Taylor Valley, Antarctica. Adams *et al*. (2006) listed a range of cosmopolitan species such as *Altenaria* sp., *Aspergillus* sp., *Cladosporium* sp. from Victoria Land. Filamentous fungi and yeasts have been discovered in drier Antarctica soils, but only yeasts are endemic to these habitats (Vishniac and Klinger, 1986; Vishniac, 1993), and filamentous fungi are mostly found in wetter coastal regions (Adams *et al*., 2006). True endemic fungi in Antarctica are thought to represent only 2-3% of the total diversity (Bridge, Spooner and Roberts, 2008b).

1.7 Role of fungi in Antarctic ecosystems

Generally fungi are known as decomposers across tropical, temperate and polar ecosystems. They play an important role in breaking down soil organic matter and releasing minerals ('mineralisation'; Swift *et al*., 1979), making them bioavailable. They also play a pioneering in colonisation of new locations and in soil structure development (Carroll and Wicklow, 1992; Wicklow and Söderström, 1997).

Psychrophilic and psychrotolerant fungi, which are typically well represented in natural communities of cold environments, also play roles such as being food spoiling agents in refrigerated and frozen foods, and can have commercial value as sources of cold-active enzymes (Margesin and Schinner, 1994). In drier Antarctic regions, fungi are the principle taxa producing sterols, which are required by soil invertebrates (Weete, 1974; Nes and McKean, 1977). They also play an important role in formation of biomass and waste decomposition in cold ecosystems (Margesin *et al*., 2007). They play further roles such as being a partner in the symbiosic relationship within lichens (Lindsay, 1978), mycorrhizal associations with *D. antarctica*, a vascular plant in Antarctica (Upson, Newsham and Read, 2008), and in mycorrhiza-like partnerships with liverworts (Williams, Roser and Seppelt, 1994), as plant pathogens in *D. antarctica* (Bridge *et al*., 2009) and in non-vascular plants, lichenicolous fungi and liverworts (Pegler *et al*., 1980). Predatory nematode-trapping fungi have been reported from Antarctica (Gray and Smith, 1984). Increasing scientific and tourism activity in Antarctica is increasing the risks of accidents such as oil spills, and fungi can play an important role in remediation of such spills (Russell, 1992; Margesin and Schinner, 1994; Aislabie *et al*., 2001; Hughes, Bridge and Clark, 2007; Gesheva, 2009).

1.8 Adaptation of fungi to the harsh environment of Antarctica

Fungi from both the Arctic and Antarctic possess adaptations allowing them to survive and thrive in the respective environments (Robinson, 2001). These include being able to remain unfrozen at sub-zero temperatures, within the range of -10°C to -15°C. Many fungi produce trehalose, a disaccharide that elevates fungal resistance towards extreme stresses such as low and high temperatures, desiccation and freezing events (Gadd, Chalmers and Reed, 1987; Hottiger, Boller and Wiemken, 1987; D'Amore, Crumplen and Stewart, 1991; Lewis, Learmonth and Watson, 1995). Trehalose is usually accompanied by other sugar alcohols and glycogen (Robinson, 2001) and can be found at higher concentrations than any other storage sugars (Thevelein, 1984). According to Niederer, Pankow and Wiemken (1992), the concentration of the trehalose doubled in excised alpine mycorrhizal roots in response to exposure to low temperatures. Fungi also produce specific antifreeze proteins that allow activity at subzero temperatures and reduce the growth of ice if crystallization does occur (Robinson, 2001). These antifreeze proteins may prevent the hyphae freezing at temperatures just below zero (Snider *et al*., 2000).

Fungi also producing exopolysaccharides (EPS), which again help to reduce their exposure to stressful conditions (Selbmann *et al*., 2002). EPS play an important role in protection from desiccation and freeze-thaw cycles in the cell (Selbmann *et al*., 2005). Lichenized fungi also utilize EPS for cellular protection (De los Ríos *et al*., 2002). In the Antarctic region, *Phoma herbarum* Cooke and the meristematic black fungus *Friedmanniomyces endolithicus* Onofri 1999 have been shown to actively produce EPS (Onofri *et al*., 1999; Selbmann *et al*., 2002; Selbmann *et al*., 2005).

Some fungi increase concentrations of glycerol and mannitol in response to heat-mediated decreases in external water potential (Cooke and Whipps, 1993). Mannitol is thought to be responsible for protection against water stress (Lewis and Smith, 1967) and for cryoprotection (Weinstein *et al*., 1997).

The membrane composition of lipids and fatty acids has an important influence on fungal ability to grow in certain temperatures ranges (Cooke and Whipps, 1993). In certain *Mucor* species, the membrane phospholipids are found to differ in thermophiles, psychrophiles and mesophiles (Hammonds and Smith, 1986), with decreasing levels of unsaturation in membrane phospholipids from psychrophiles to mesophiles and to thermophiles, thereby ensuring that membrane fluidity is maintained.

Fungi also have some adaptation strategies towards UV radiation stress. According to Arcangeli *et al*. (1997) and Arcangeli and Cannistraro (2000) *Arthrobotryx ferox* Onofri and S. Tosi, a nematophagus and entomaphagus Antarctic fungus, produces carotenoids and mycosporins to protect from UV radiation. In their symbiotic relationship within lichens, fungi with dark pigments play an important role by screening harmful UV radiation protecting both the fungal and algal cells (Armstrong, 2003). Other examples of species with UV resistance include *Stachybotrys chartarum* (Ehrenb.) S. Hughes 1958 and *Ulocladium consortiale* sensu Brook (Domsch, Gams and Anderson, 1980). However, even though fungi possess adaptations towards high UV radiation, low water availability and low temperature, their real limits of growth and survival are generally still unknown (Onofri *et al*., 2004).

Other groups of fungi showing specific adaptation towards the extreme environmental stresses of Antarctica are the black fungi and cryptoendolithic communities (Onofri *et al*., 2008). Cryptoendolithic communities are the only life existing in the most extreme environments with driest, coldest and harshest UV radiation conditions (Onofri *et al*., 2008). This group of fungi has been exposed to the real space environment in the

International Space Station to test the ability of life to survive under extreme extraterrestrial conditions such as on Mars (Onofri *et al*., 2008).

Enzymes in Antarctic fungi also show adaptation to the extreme environment. This is achieved by having reduced activation energies (Miyazaki *et al*., 2000). However, while their catalytic efficiency increases at low temperature these enzymes are also denatured at higher temperatures (Margesin *et al*., 2007). Enzymes of psychrophilic and psychrotolerant microorganisms also have to increase the flexibility of their proteins in order to compromise reduced thermal energy in the low temperature environment (Somero, 2004).

Overall, the physiological adaptation of fungal assemblages to a range of environmental stresses is complex, which means that all the cell components have to function together in order to adapt to low temperature (Robinson, 2001).

1.9 Substrata for fungi in the Polar Regions

Keratin is a substrate which can be found in materials like feather, hair and wool (Gushterova *et al*., 2005). Fungi that are able to degrade keratin are known as keratinophilic, and are mostly found in the areas influenced by plants and animals (Marshal, 1998). The presence of keratinophilic fungi has been reported on Antarctic Peninsula (Caretta and Piontelli, 1977) and Signy Island (Pugh and Allsop, 1982). *Geomyces vinaceus* Dal Vesco, and *Chrysosporium merdarium* (Ehrenb.) J.W. Carmich. 1962 are two examples of keratinophilic fungi that have been reported from Antarctic soil by (Mercantini, Marsella and Cervellati, 1989). *Geomyces pannorum* (Link) Sigler & J.W. Carmich. 1976 is another example of a keratinophilic fungus that has been reported by Finotti *et al*. (1996).

Cellulolytic fungi feed on cellulose, which is the most abundant substance on the Earth (Chi *et al*., 2009), especially in plant biomass and cell walls (Percival Zhang, Himmel, and Mielenz, 2006). This carbohydrate group is degraded by cellulase to produce glucose (Highley, 1997). Cellulolytic fungi have been isolated from South Georgia (Smith, 1981), Syowa Station (Yamamoto *et al*., 1991), Victoria Land (Fenice *et al*., 1997) and the Ross Sea region (Duncan *et al*., 2008).

Amylolytic fungi in the Antarctic region play a role in degrading starch or amylose. It breaks long starch chains into small amylose or glucose chains. Reports of amylolytic fungi from the Antarctic are very limited, from Victoria Land (Fenice *et al*., 1997) and continental Antarctic ice (Margesin *et al*., 2003).

Protein provides another type of enzyme substrate. Proteolytic fungi, which produce protease to degrade protein into amino acids and polypeptides, have been reported (Tosi *et al*., 2001; Margesin *et al*., 2003), including specifically from the Schirmacher Oasis (Ray *et al*., 1992), Victoria Land (Fenice *et al*., 1997) and Windmill Island (Bradner *et al*., 1999a).

Animal and bird guano provide a further substrate in this extreme environment, utilised by coprophilic fungi. *Thelebolus microsporus* (Berk. & Broome) Kimbr. 1967 is a coprophilic fungus reported from the Antarctic region (Leotta *et al*., 2002). There are also reports of coprophilic fungi from Bird Island, sub-Antarctic (Bridge, Hughes and Denton, 2008) and Hope Bay (Leotta *et al*., 2002).

1.10 Previous reports of fungal diversity from Antarctica

To date, more than 1000 species of fungi have been described from the Antarctic region including the sub-Antarctic, maritime Antarctic and continental Antarctic (Bridge *et al*., 2009). Reports of fungi from continental Antarctica includes aspects of biodiversity

(Selbmann *et al*., 2005; Held *et al*., 2005; Arenz *et al*., 2006; Fell *et al*., 2006; Blanchette *et al*., 2010), biochemistry (Mercantini *et al*., 1993; Aislabie *et al*., 2001; Duncan *et al*., 2008; Arenz *et al*., 2010; Duncan *et al*., 2010; Arenz and Blanchette, 2011), taxonomy (de Hoog *et al*., 2005), molecular biology (Bradner *et al*., 2003; Lawley *et al*., 2004; Malosso *et al*., 2006) and biotechnology (Margesin *et al*., 2007).

Reports from the maritime Antarctic again include molecular aspects (Jumpponen, Newsham and Neises, 2003; Lawley *et al*., 2004; Malosso *et al*., 2006; Bridge and Newsham, 2009), biochemistry (Hughes, Lawley and Newsham, 2003; Hughes *et al*., 2007; Kasieczka-Burnecka *et al*., 2007; Tosi *et al*., 2010) and biodiversity (Marshall, 1998; Bridge and Worland, 2004; de Hoog *et al*., 2005; Gushterova *et al*., 2005; Xin and Zhou, 2007; Newsham and Bridge, 2010).

Reports from the sub-Antarctic are very limited. There are reports from Bird Island (Bridge *et al*., 2008a), South Georgia (Newsham, 2010), Iles Kerguelen (Pegler *et al*., 1980) and Macquarie Island (Kerry, 1990a).

Even though there are more individual studies based in continental Antarctica in comparison with the maritime Antarctica, the species richness reported from the latter region is considerably higher (Bridge *et al*., 2009; Tosi *et al*., 2010).

1.11 Enzymes obtained from microbes in Antarctica

Microbes inhabiting extreme environments have evolved some unique survival mechanisms, including enzyme production (Margesin *et al*., 2007). Enzymes are proteins which help organisms to break down the food they consume by catalysis (DeSantis, 1983), acting either intracellularly or extracellularly. Enzymes obtained from microbes in

Antarctica usually show psychrophilic or psychrotolerant characteristics. These psychrophilic and psychrotolerant enzymes can effectively function at low temperatures (Feller *et al*., 1996; Feller and Gerday, 2003) and posses several advantages for applications in the biotechnology industry, for instance in saving energy, shortening the processing time and reducing contamination risks (Margesin *et al*., 2007).

Psychrotolerant enzymes have higher catalytic activity below 40°C (Nichols *et al*., 2002). Pychrophilic enzymes have been studied widely from bacterial sources in various contexts especially cloning, sequencing and crystal structure (Russell, 2000). For instance, α-amylase has been cloned and sequenced from *Alteromonas haloplanctis* and its crystal structure has been studied (Russell, 2000). This is the first psychrophilic enzyme to be crystallized from Antarctic region (Aghajari *et al*., 1996).

Previous studies of Antarctic material have examined enzymes from actinomycetes from Windmill Island (Gesheva, 2009), Livingston Island (Gushterova *et al*., 2005), algae from Casey (Irving *et al*., 2005), locations in the continental and maritime Antarctic (Sabacka and Elster, 2006), and in Antarctic sea ice (Wang *et al*., 2009) and bacteria (Gesheva, Stackebrandt and Vasileva-Tonkova, 2010; Peters *et al*., 2010). Enzymes produced by cold region microbes have been used in biotechnology, taking advantage of their high catalytic activity at low temperature (Nakagawa *et al*., 2006; Margesin *et al*., 2007). Mesophilic counterpart enzymes tend to have reduced catalytic activity at higher temperatures (Morita *et al*., 1997).

Enzymes that have been isolated from Antarctica and used in applications in biotechnology are listed in the Table 1.1. Hydrolase enzymes have a major role in biotechnological applications. Among the six groups of enzyme listed, there are no reports of the transferase group in Antarctica. Other groups of enzyme, oxidoreductase, lyase, isomerase

and ligase, have very limited numbers of reports. Therefore the hydrolase group contributes the majority of reports from the Antarctic region.

Table 1.1: Enzymes and their application

Enzyme classification	**Enzyme**	**References**	**Application**
Oxidoreductase	Isocitrate dehydrogenase	Sahara *et al*. (1999)	Selective, sensitive, and rapid on-line monitoring of low-temperature processes and quality control
	Lactate dehydrogenase	Mitchell, Yen and Methemeier (1985)	
	Catalase	Gocheva *et al*. (2009); Gesheva *et al*. (2010)	Cold pasteurization and food preservation
	Oxidase	Fenice *et al*. (1997); Gesheva *et al*. (2010)	Cold pasteurization, food preservation
Hydrolase	Amylase	Fenice *et al*. (1997); Gesheva (2009)	Contact lens cleaning, biopolishing
	Protease	Ray *et al*. (1992); Fenice *et al*. (1997); Gesheva (2009)	Low temperature washing, meat tenderization, removal of fish skin, synthesis of volatile and heat sensitive compounds,
	Cellulase	Yamamoto *et al*. (1991); Fenice *et al*. (1997); Gesheva (2009)	Contact lens cleaning, juice clarification, protoplast formation, stone washing fabric
	β-Galactosidase	Margesin *et al*. (2003); Song *et al*. (2010)	Lactose hydrolysis in milk product
	Keratinase	Gesheva (2009); Shih (1993); Onifade, Al-Sane, Al-Musallam and Al-Zarban, (1998)	Poultry and leather industry (

Table 1.1 continued.

	DNase	Gesheva (2009)	Molecular biology
	Lipase	Fenice *et al*. (1997); Margesin *et al*. (2003)	washing at low temperatures, synthesis of volatile and heat sensitive compounds and flavourings
	Xylanase	Bradner *et al*. (1999b); Scorzetti *et al*. (2000)	Used in the baking industry
	Chitinase	Fenice *et al*. (1997); Bradner *et al*. (1999b)	To control plant pathogens, additives in antifungal cream and drugs
	Esterase	Soror *et al*. (2007)	Synthesis of volatile and heat sensitive compound
	β-lactamase	Margesin *et al*. (2003); Gesheva (2009); Gesheva (2010)	Antibiotic degradation
	Phosphatase	Fenice *et al*. (1997); Gesheva *et al*. (2010)	Molecular biology
Lyase	Pectate lyase	Margesin, Fauster and Fonteyne (2005)	Clarification of juice
Ligase	Antioxidant enzymes	Gocheva *et al*. (2009); Wang *et al*. (2009); Tosi *et al*. (2010)	Cosmetic products

Adapted from Margesin *et al*. (2007) and Kasieczka-Burnecka *et al*. (2007)

1.12 Research on hydrolase enzymes in the Antarctic region

According to Enzyme Nomenclature, International Union of Biochemistry and Molecular Biology (IUBMB), hydrolase enzymes are categorized in group 3. Hydrolase enzymes categorized as such because they catalize hydrolysis of certain target chemical bonds (Bornscheuer and Kazlauskas, 2006).

Fungi are able to produce various enzymes depending on the environment in which they exist. They are well known to produce certain metabolites and also enzymes (Singh, Puja and Bhat, 2006). Therefore, fungi from Antarctica can be considered as a major source of psychrophilic and psychrotolerant enzymes.

Investigation of enzymes from filamentous Antarctic fungi are limited to a few studies (Fenice *et al*., 1997; Bradner *et al*., 1999a, b; Kasieczka-Burnecka *et al*., 2007). The majority of the existing reports are from continental Antarctica rather than the maritime Antarctic (Margesin *et al*., 2007). It is also noteworthy that a majority of enzyme studies from the Antarctic region have focused on bacteria and rarely on yeasts (Birgisson *et al*., 2003). In this study we address this by exploring King George Island for its diversity of soil fungi and their extracellular hydrolase enzyme properties.

1.12.1 Amylase

Amylases can be classified as α-amylase (E.C.3.2.1.1), β-amylase (E.C.3.2.1.2) and γ-amylase (E.C.3.2.1.3). Generally, amylases are responsible for the hydrolysis of cleavage β-1, 4 linkages of starch and glycogen (Highley, 1997). α-amylase is responsible for the hydrolysis of α-1, 4- linkages to produce oligosaccharides, maltose and glucose (Joseph *et*

al., 2007). β-amylase produces maltose by attacking the second α-1, 4 glycosidic bond. The last subclass, glucoamylase, produces glucose by attacking the last α-1, 4 glycosidic bond (Joseph *et al*., 2007). Amylase has been widely used in various fields for instance, to saccharify starch in order to produce ethanol in brewing, to remove starch stains from fabrics and to produce maltose-containing syrup in the baking industry (Joseph *et al*., 2007). Amylase has been screened from actinomycete strains which were isolated from soils of Casey Station or Dewart Island (Gesheva, 2010) and Terra Nova Bay (Gesheva, 2009), fungal strains from Victoria Land (Fenice *et al*., 1997) and bacteria and yeast (Margesin *et al*., 2003). Commercially, amylase from fungi is currently normally obtained from *Aspergillus oryzae* (Joseph *et al.*, 2007).

1.12.2 Cellulase

Cellulase is an enzyme which is responsible in degrading cellulose (Joseph *et al*., 2007). This enzyme is an extracellular enzyme and attacks β-linked glucosyl units in cellulose to produce the simpler sugar, glucose (Duncan *et al*., 2008). Cellulase can be categorized as Endo-1, 4-β-glucanase (EC 3.2.1.4), Exo-1, 4-β-glucanases and β-D-glucosidase. Endo-1, 4-β-glucanase catalyses the hydrolysis of β 1, 4-glusidic links (Duncan *et al.*, 2008). This enzyme has been used in the textile industry such as in stone the washing process of denim jeans, to increase efficiency in paper making processes, the clarification of juice and in biopolishing (Margesin *et al*., 2007). The latter is used for cotton garments in restoring softness and reducing pill formation (Marx *et al*., 2006). Cellulase has been detected from actinomycete strains from Casey Station (Gesheva, 2010), Victoria Land (Gesheva, 2009) and fungi from Ross Island (Duncan *et al*., 2008), Syowa Station

(Yamamoto *et al*., 1991) and Victoria Land (Fenice *et al*., 1997). Commercially cellulase is normally obtained from *Aspergillus* and *Trichoderma* (Joseph *et al*., 2007).

1.12.3 Protease

Protease hydrolyses proteins into polypeptides and amino acids (DeSantis, 1983; Rao *et al*., 1998). Protease from microorganisms dominates the enzyme industry (Outtrup and Boyce, 1990), and the most important sources of these enzymes are fungi and bacteria (Manivannan and Kathiresan, 2007). Protease has been screened from actinomycetes from Casey Station (Gesheva, 2010) and Victoria Land (Gesheva, 2009), fungi from Victoria Land (Fenice *et al*., 1997), Windmill Island (Bradner *et al*., 1999a) and Schirmacher Oasis (Ray *et al*., 1992) and bacteria from the Vestfold Hills (Nichols *et al*., 2002). Psychrophilic proteases can be used to save energy (Feller and Gerday, 2003). For instance, psychrophilic protease can be used in the industrial peeling of leather in normal tap water temperature, not requiring energy to heat to the 37˚C required by mesophilic enzymes (Feller and Gerday, 2003). Proteases are also used to tenderize meats in food industries (Marx *et al*., 2006). This class of enzyme has also been widely used in other industrial processes such as dry cleaning, detergent, cheese making, silver recovery from photographic film and many others (Nout and Rombouts, 1990).

1.13 Other hydrolase enzymes with potential application in biotechnology

Enzymes such as subtilisin, lipase and glycosidases, which are added to detergents to remove macromolecular stains, are poorly active at normal tap water temperatures. This

problem can overcome by the use of cold active enzymes (Feller and Gerday, 2003). Forty percent of the market for enzymes in the detergent industry is represented by cold-adapted enzymes such lipases, amylases, proteases and cellulases (Marx *et al*., 2006). β-galactosidase, which catalyses the hydrolysis of lactose into glucose and galactose, have an important role in the removal of lactose from milk. This is helpful to enhance digestibility and avoid allergy problems caused by lactose (Nakagawa *et al*., 2003; Feller and Gerday, 2003). Cold active β-galactosidase has high catalytic activity at below 20ºC (Coker *et al*., 2003).

Chitinase is an important enzyme in the degradation of chitin (Mavromatis *et al.*, 2003). It can be obtained from crab shells and has an important value in the medical field, such as being a potential additive in antifungal creams and lotions and antifungal drugs (Dahiya, Tewari and Hoondal, 2006). Pectinase, another cold-active enzyme, can be used in the fruit juice industry, enhancing extraction yield, clarification and taste (Marx *et al*., 2006). Glycosidase, an enzyme widely used in the baking industry, can be substituted by psychrophilic glycosidases to avoid remaining residual activity after cooking which will affect the final product during storage (Margesin and Schinner, 1999).

Antarctic fungi have been investigated in the context of biodiversity studies, but there remain very few reports from maritime Antarctica. Furthermore, specific investigations of extracellular hydrolase enzymes from maritime Antarctica have largely not been attempted, although a small number of reports exist from continental Antarctica. This lack of information on soil microfungal diversity and enzyme studies from maritime Antarctica motivated us to:-

1. To analyse the diversity of soil fungi of the Fildes Peninsula, King George Island, maritime Antarctic using soil plating method
2. To screen for activity of extracellular hydrolase enzymes (amylase, cellulase and protease) of the fungi isolated; and
3. To quantify amylase and cellulase activities

2.0 Materials and Methods

Culture media, reagents and chemicals and their composition, preparation and other conditions are given in Appendices A - B

2.1 Diversity of soil microfungi from Fildes Peninsula, King George Island

2.1.1 Soil sampling

King George Island is the largest island of the South Shetland Island archipelago. Three quarters of the island's area is covered by ice or permanent snow, with the remainder being ice-free. This island has a diverse flora and fauna, including seals, penguins, seabirds, arthropods, fungi, algae, moss, bacteria and the two native Antarctic flowering plants *D. antarctica* and *C. quitensis.*

Soil samples were collected during the 2006/7 austral summer (February 2007) near the Chilean scientific research station, Prof. Julio Escudero, which is located on the Fildes Peninsula, King George Island (62º 12' 57"S, 58º 57' 35"W) (Figure 2.1a, b, c). The samples were collected from various habitats (n = 5; Figure 2.2a, b, c, d), including two ornithogenic sites, two human-impacted areas (lake and station) and a glacier foreland (pristine) (Figure 2.1c).

Each sampling point location was recorded using a hand held Global Positioning System (GPS). Prior to collection of soil samples, physical parameters (temperature and pH) were recorded at each collection site using a Hanna Instruments pHep4® Waterproof pH

Tester and thermometer. Summary descriptions and GPS locations of collection sites are given in Table 2.1.

To collect soil samples (n = 5 samples per location), the top 0-10cm of the soil profile was sampled using a sterile spatula, giving approximately 10 g of soil, which was stored in sealed sterile whirl packs or Falcon tubes. After collection, samples were rapidly returned to the research station, where they were refrigerated at 4°C, subsequently being transported at the same temperature to the National Antarctic Research Center, Kuala Lumpur.

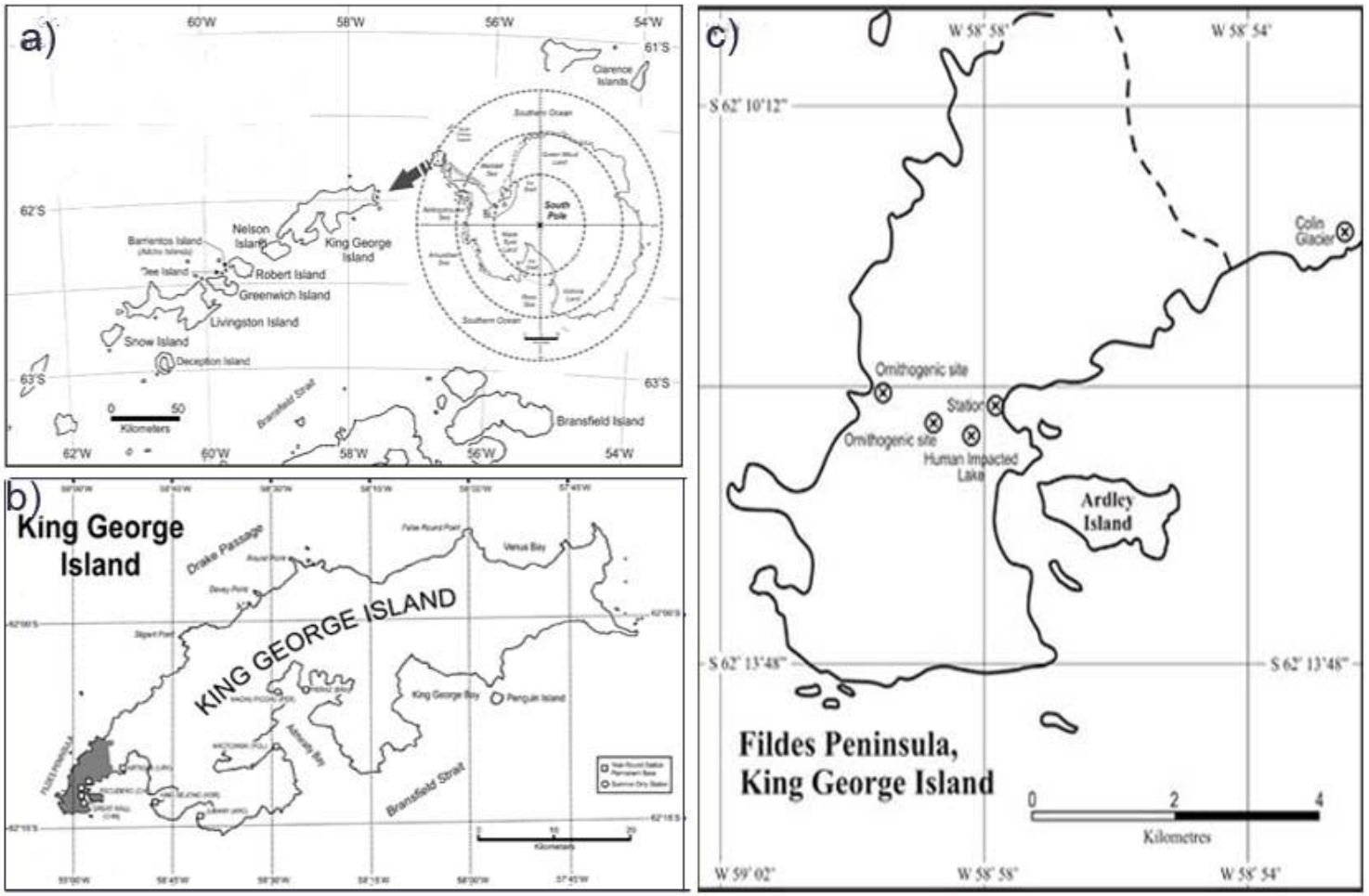


Figure 2.1: Location of sampling sites a) Location of South Shetland Islands in Antarctica b) Enlargement of King George Island c) sampling sites on the Fildes Peninsula, King George Island

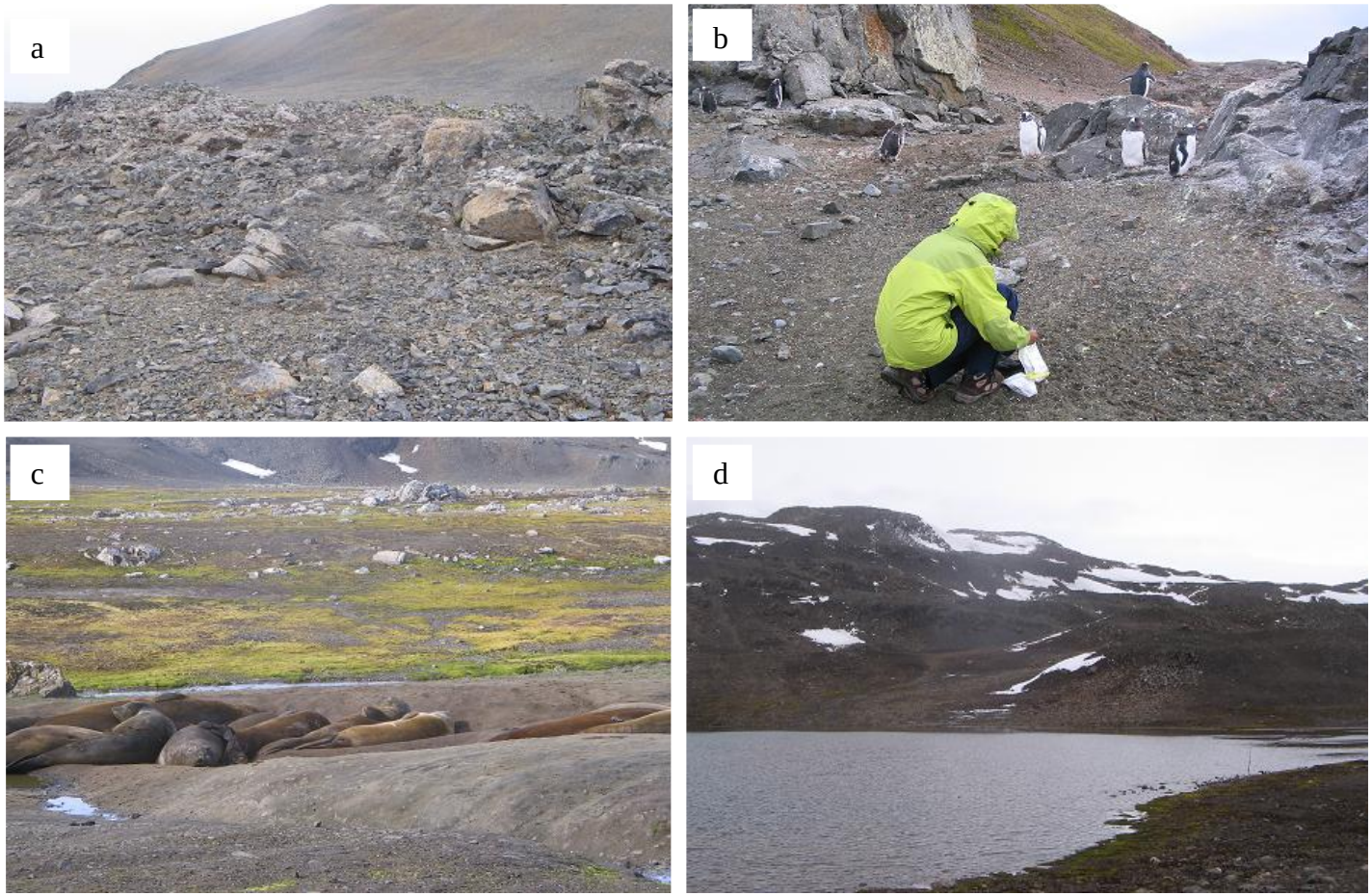


Figure 2.2: Sample collection sites on the Fildes Peninsula, King George Island. a) Ornithogenic site 1 b) Ornithogenic site 2 c) vertebrate impacted site d) human impacted lake shore

Table 2.1: Summary description of soil sample collection sites, and environmental conditions at the time of collection on the Fildes Peninsula, King George Island

Sample ID	Habitat notes	GPS	Temperature			
			Soil ºC	Surface ºC	Air ºC	Water ºC
AK07KGI1 Human impacted lake shore	Highly contaminated Small lake nearby Snow patch	S62º12'14.9" W58º58'00.8"	3.0	6.3	5.3	7.5
AK07KGI2 Ornithogenic site	On the hill On rock surface Feathery	-	-	7.0	6.7	-
AK07KGI3 Ornithogenic site	Near to sea side Surrounded by seal Feathery	S 62º11'51.6" W58º59'28.8"	6.0	7.6	5.1	10.0
AK07KGI10 Glacier foreland	Glacier Sea side Muddy soil	S62º10'03.2" W58º51'16.2"	8.9	8.8	5.3	7.8
AK07KGI20 Station site	Behind Escudero Hill Slopy, rocky	S62º12'57" W58º57'35"	3.9	4.2	4.1	-

2.1.2 Fungal isolation and identification

Soil fungi were cultured on Potato Dextrose Agar (PDA) media according to Warcup's (1950) soil plating method. Approximately, 0.1g soil was placed in a sterile petri dish and sterilized PDA medium supplemented with chloramphenicol (0.2g/l) was poured into it. PDA is an effective medium for the isolation of fungi and is widely used in fungal research for that purpose (Azmi and Seppelt, 1997). Replicate plates (n = 6) were prepared, with three incubated at 4°C and three at 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 morphological characteristics such as colony colour, fruiting body and size of the spores (Barnett and Hunter, 1972; Onofri *et al*., 2007b). Fungal strains were deposited in the Institute of Biological Science, University Malaya, fungal collection which is located in National Antarctic Research Center, Kuala Lumpur.

The six fungal isolates found to be the most effective enzyme producers (see section 2.2) were then grown in PDA broth in a 4°C orbital shaker for 10 days. The fungi were freeze-dried prior to DNA extraction at the National Taiwan Ocean University by our collaborator, Dr. Ka Lai Pang. Genomic DNA was extracted using the DNeasy Plant DNA Extraction Kit (Qiagen) 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 of each primer, 0.2 mM of each dNTP, 2.5 mM $MgCl_2$ and 1.25 U of *Taq* Polymer-

ase (Invitrogen). The amplification cycle consisted of an initial denaturation step of 95°C for 2 min followed by 35 cycles of (i) denaturation (95°C for 1 min), (ii) annealing (54°C for 1 min) and (iii) elongation (72°C for 1.5 min) and a final 10 min elongation step at 72°C. The PCR products were analysed by agarose gel electrophoresis and 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. While this generates a number of 'nearest neighbour' sequence identities from those present in the database, it is important to note that this provides only a coarse suggestion of isolate identity, even where named species are linked, not least as robust taxonomic knowledge of Antarctic fungi (as with all microbial groups) and their relationship with non-Antarctic taxa is currently unavailable.

2.1.3 Formulae and Diversity Indices

Frequency of occurrence of soil microfungi from Fildes Peninsula, King George Island was calculated using the following formula:

$$Frequency\ of\ occurrence = \frac{Number\ of\ occurrence}{Number\ of\ plates} x\,100$$

The taxa with frequency of occurrence 30% or more defined to be the most common. Those with frequency of occurrence between 10% and below 30% are defined as common taxa and those with frequency of occurrence less than 10% defined as less common taxa.

The Margalef Diversity Index was used to calculate species richness, using the following formula:

$$\text{Margalef Diversity Index} = \frac{S-1}{\ln(n)}$$

S- Total number of taxa
n- number of species in a specific group

Species diversity and evenness were calculated using the Shannon Wiener Diversity Index:

$$H = -\sum Pi\ (\ln\ Pi)$$

H- Shannon Wiener Index

Pi- Total number of species

$$E = H/\log(S)$$

E- Evenness

H- Shannon Wiener Index

S- Total number of species in the community/richness

2.2 Screening of extracellular hydrolase enzymes from psychrophilic and psychrotolerant fungi from Fildes Peninsula, King George Island

2.2.1 Media Preparation

Extracellular hydrolase enzyme activity was screened as described by Margesin *et al*. (2003). The presence of amylase, cellulase and protease activity was tested 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 starch (Cat number: S9765 Sigma Aldrich) (0.4% w/v), carboxymethylcellulose (Cat number: 419338 Sigma Aldrich) and trypan blue (Cat number: 76146 Sigma Aldrich) (0.4% and 0.01% w/v), or skim milk powder (0.4% w/v) respectively. The ingredients for media were mixed together and sterilized at 121°C for 20 min. The medium was left to cool and poured into petri dishes. Solidified media were kept in a refrigerator at 4°C until required.

2.2.2 Screening of amylase, cellulase and protease

All test fungi were subjected to the agar plug assay method and prepared in three replicates. Agar plugs (6 mm) were bored from the growing edge of the fungal colonies on the PDA plates using cork borer number 3 and inoculated into a 6 mm well made at the centre of each assay agar plate. Plates were then incubated at 4°C. The assay agar plates were prepared in triplicates. After 10 d, the plates were examined for the presence of a clear zone in the agar around the colony, indicating extracellular enzyme activity. Yeast sp.24 was used as a control since it has no activity for any of the enzyme screened. Amylase and

protease activities were confirmed by staining the plates with Lugol's solution and Coomassie brilliant blue solution respectively.

2.2.3 Relative enzyme activity (RA)

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 dimensions, at 90° perpendicularly, and the mean value calculated. The activity zone was determined by calculating 'relative enzyme activity' (RA) using the following formula:

$$\text{Relative enzyme activity} = \frac{\text{Clear zone diameter} - \text{colony diameter}}{\text{Colony diameter}}$$

Following Bradner *et al*. (1999a) and Duncan *et al*. (2008) strains exhibiting an RA of > 1.0 were classified as having 'significant activity'.

Bradner *et al*. (1999a) used relative enzyme activity (RA) to measure enzyme activity. However they did not determine the cutting point of a good enzyme producing fungi. Therefore we followed Duncan *et al*. (2008), to decide relative activity (RA) more than 1.0 possess significant activity.

2.3 Extracellular hydrolase enzyme quantification from selected soil microfungi

2.3.1 Amylase production

The strongest three amylase producing isolates (Table 2.2) were chosen and subjected to amylase assay. An amylase production medium which contained soluble starch as the sole carbon source was prepared, pH adjusted to 6.5, and then distributed to nine 250ml flasks. These were sterilized at 121°C for 20 min in an autoclave. The sterilized media were stored at 4°C until required. Selected fungal mycelia grown on PDA media were cut using cork borer number 3 (6mm diameter plug) and inoculated into the prepared amylase production medium. This was prepared in triplicate. Flasks were incubated at 4°C under shaking conditions (100 rpm) for 10 d.

2.3.1.1 Crude amylase

After incubation, each flask was filtered (Whatman no.1 filter paper) to separate the mycelia from the liquid medium. The filtrate contained the crude amylase. Each flask was filtered and processed separately, with the activity values obtained then being averaged. The individual flask filtrates were poured into 50ml tubes and centrifuged at 4000 rpm for 30 min at 4°C to obtain a cell-free culture. The supernatant was stored at -20°C until required.

The total protein content and amylase activity in the crude culture were assayed using the Bradford Protein Assay (Bradford, 1976) and reducing sugar method (Miller, 1959), respectively (see sections 2.3.3 and 2.3.4). Specific activity of the enzyme was calculated

by dividing glucose production by protein content and expressed in U/mg. One unit of enzyme is defined as the amount required to liberate 1 µmol of glucose.

2.3.2 Cellulase production

The three strongest cellulase producing strains were chosen for cellulase assay (Table 2.2). A cellulase production medium, Mandels salt medium (Mandels and Reese, 1957), containing carboxymethylcellulose (CMC) as the only carbon source was prepared in a 1000 ml erlenmeyer flask. The pH of the medium was adjusted to 6.5 and it was distributed to nine 250 ml conical flasks, each containing 100ml. These were sterilized at 121°C and 15 psi for 20 min and stored until further use.

The sterilized media were inoculated with plugs of fungal mycelium grown on PDA, which was cut using cork borer number 3 (6mm diameter) and prepared in triplicate, and incubated at 4°C for 10 d under shaking conditions (100 rpm).

2.3.2.1 Crude cellulase

The culture medium was filtered (Whatman No.1 filter paper), using a filter pump. The filtrate contained crude cellulase. This crude cellulase was poured in 50ml centrifuge tubes, and centrifuged at 4000 rpm for 30 min at 4°C to separate remaining pellets from the liquid medium, and stored at -20°C until required.

The total protein content and cellulase activity in the crude culture were assayed using Bradford Protein Assay methods (Bradford, 1976) and Miller (1959) (see sections 2.3.3 and 2.3.4). The specific activity of the enzyme was calculated by dividing glucose produc-

tion by protein content and expressed in U/mg. One unit of enzyme is defined as the amount required to liberate 1 μmol of glucose.

During assays, enzymes were incubated at 50°C because five of the six strains used in this study were psychrotolerant, and such enzymes tend to have a wide range of active temperature. This temperature also has been used previously in measurement of enzyme activity of Antarctic fungi (Bradner *et al*., 1999a; Duncan *et al*., 2008).

Table 2.2: The strongest three enzyme producing strains for each enzyme type examined

Enzyme	Taxon	Strain
Amylase	*Geomyces pannorum*	AK07KGI2001 R2-1 Station site
	Geomyces pannorum	AK07KGI102 R1-4 Human impacted lake shore site
	Geomyces pannorum	AK07KGI301 R3-3 Ornithogenic site
Cellulase	*Mrakia frigida*	AK07KGI103 R2-1 Human impacted lake shore site
	Geomyces pannorum	AK07KGI1001 R1-2 Pristine site
	Geomyces pannorum	AK07KGI2001 R2-1 Station site

2.3.3 Bradford Assay

Total protein concentration of the crude enzyme was measured using the Bradford Protein Assay Method (Bradford, 1976).

2.3.3.1 Standard stock solution

A standard protein solution was prepared by mixing 1mg of Bovine Serum Albumin (BSA) with 1 ml of ddH_2O. This was made up to 10ml with sterile distilled water and 1 ml aliquots added to each of 10 microcentrifuge tubes, and stored at -20°C until further use. This standard solution is stable for several months.

2.3.3.2 Standard curve

Nine test tubes were prepared in order to generate the standard curve. The standard stock solution was diluted to eight different concentrations, and a further control was prepared without protein. 100µL of each of these dilutions was dispensed into separate test tubes to which was then added 5ml of Bradford reagent. This mixture was vortexed before absorbance was measured using a Shimadzu UV-160A Spectrophotometer at 595nm. A graph of OD versus % protein concentration was plotted. (Figs. 2.3, 2.4). The total protein concentration of the crude enzyme extracts described above was determined from these standard curves.

2.3.3.3 Total protein assay

The assay tubes were prepared in triplicate. 5ml of the Bradford reagent was added to 100µL of crude enzyme extract and vortexed. The OD of this mixture was then read using the Shimadzu UV-160A spectrophotometer at 595nm. The total protein content in the crude amylase was determined using the standard curve (see section 2.3.3.2).

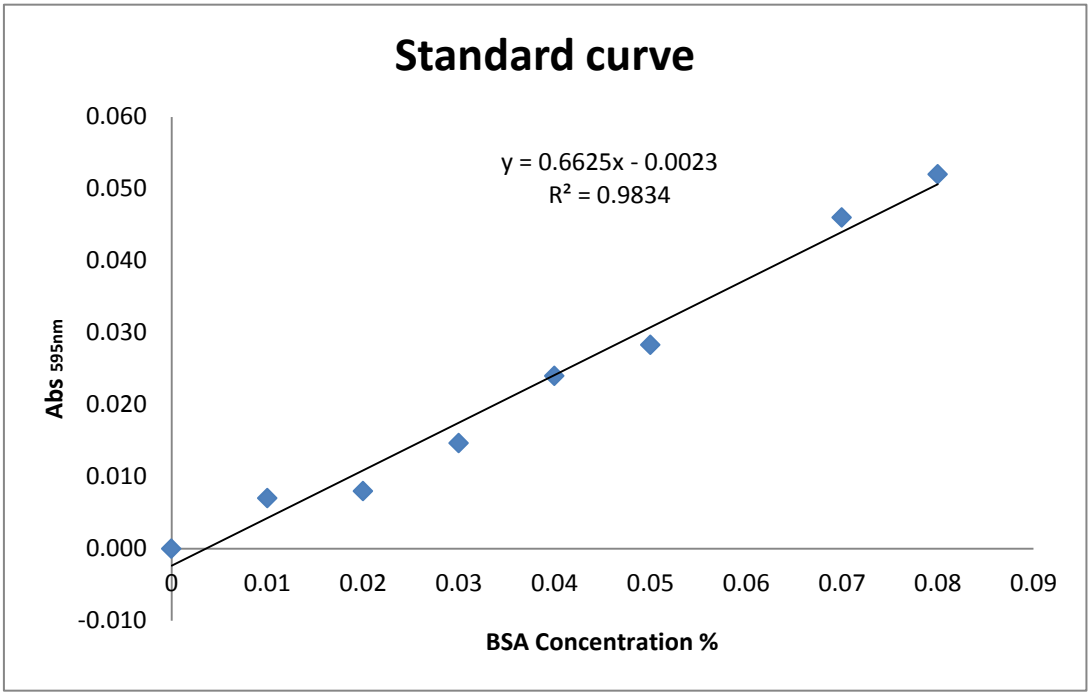


Figure 2.3: Standard curve for total protein content for amylase

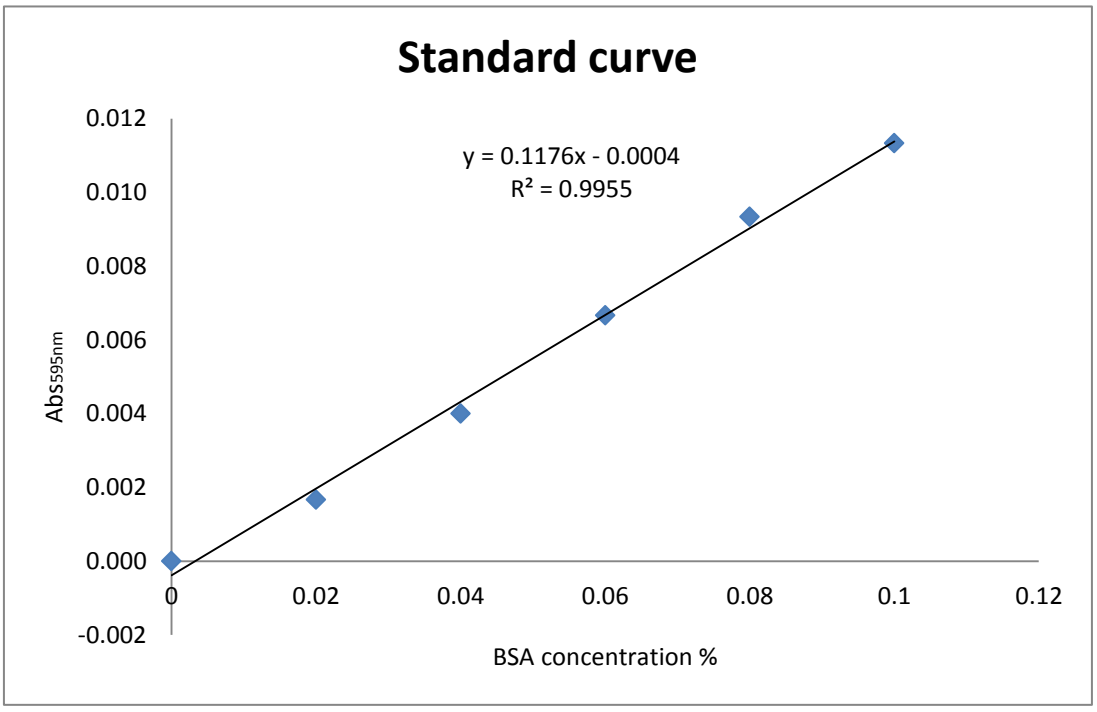


Figure 2.4: Standard curve for total protein content for cellulase

2.3.4 Reducing sugar

2.3.4.1 Enzyme assay

1.0 ml of 1% carboxymethylcellulose (CMC) (Sigma Aldrich cat number: 419338) in citrate phosphate buffer (Cat number: 2851 Sigma Aldrich) (pH 6.5) was dispensed in a test tube. Then 1.0 ml of the enzyme solution was added to the test tube which was incubated at 50°C (Bradner *et al*., 1999a; Duncan *et al.*, 2008) in a water bath for 60 min. At the end of this period, the enzyme reaction was stopped by adding 3.0 ml of DNS solution and the mixture was vortexed.

2.3.4.2 Blank and control

2.0 ml of citrate phosphate buffer (0.1M) added in a separate test tube as a blank. An enzyme control and a substrate control were prepared. For the enzyme control, 1.0 ml of enzyme solution and 1.0 ml of citrate buffer were added together in a test tube. In a separate test tube, 1.0 ml of substrate solution and 1.0 ml of citrate buffer were added together as substrate control.

2.3.4.3 Glucose standard

A stock solution of anhydrous glucose (R&M Chemicals cat number:50-99-7) was made up (10mg/ml) and dispensed into the micro centrifuge tubes. The aliquots were then stored at -20°C until further use. Standard mixtures were vortexed after thawing, prior to use. 1.0 ml of the standard solution was then added to 1.0 ml of citrate buffer, which had

already been dispensed in a test tube. The enzyme assay, blanks, controls and standard were incubated together at 50°C in a water bath for 60 min. At the end of this period the reaction was stopped by adding 3.0 ml of DNS reagent. All the tubes were then boiled for 5 min and placed in a cold water bath. Then 0.2 ml of the mixture was added to 2.5 ml distilled water. The OD of the coloured mixture was measured in a Shimadzu UV-160A spectrophotometer at 540nm.

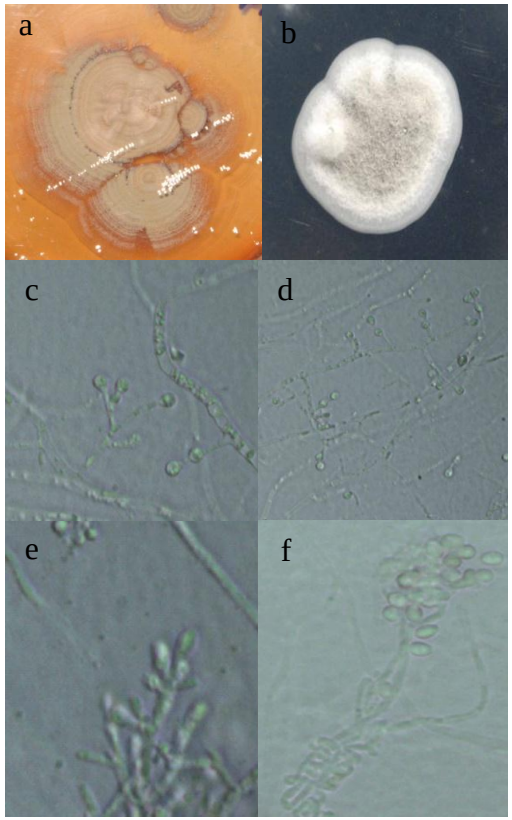
3.0 Results

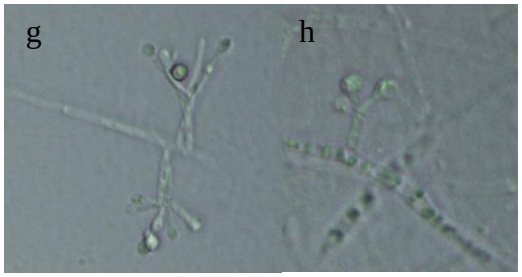
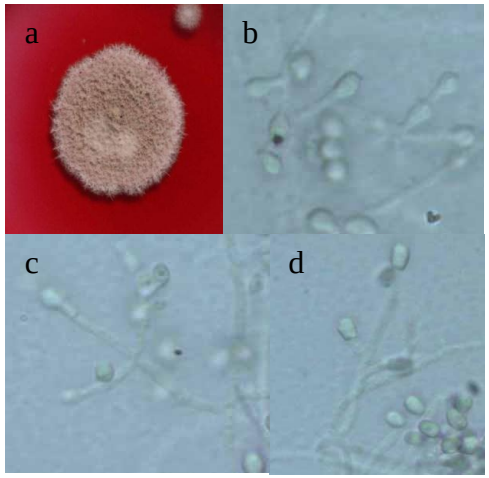
3.1 Diversity of fungi

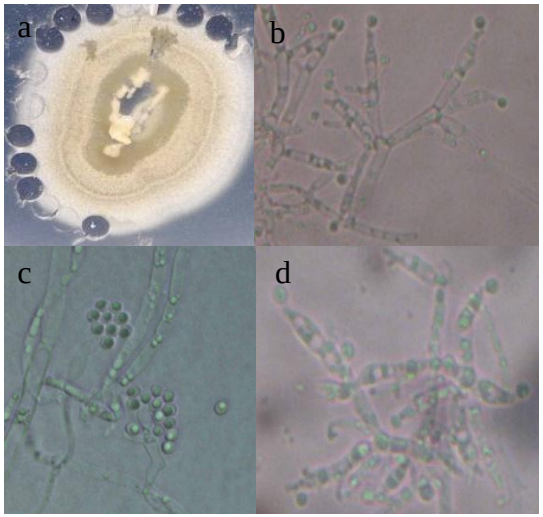
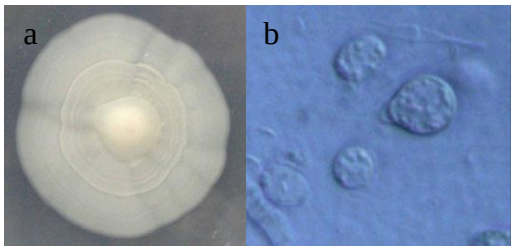
3.1.1 Morphological description

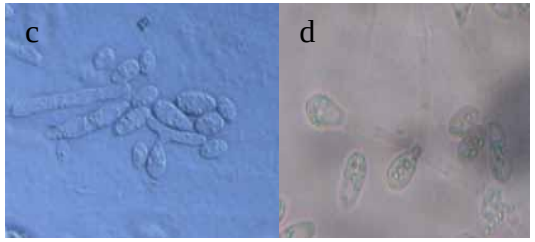
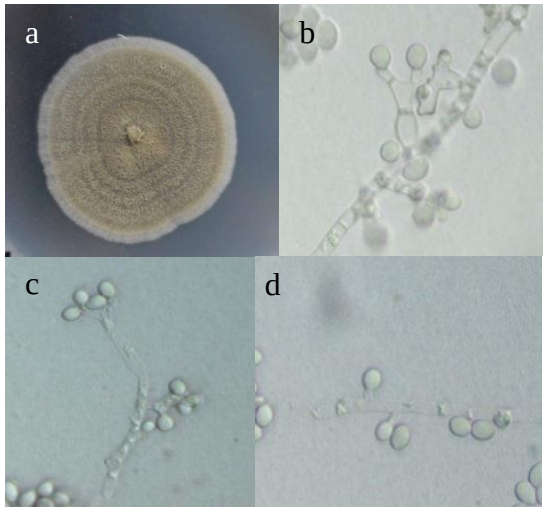
Forty-one morphologically distinct fungal strains were isolated in culture from five soil samples which were collected from the Fildes Peninsula, King George Island, maritime Antarctic. This included 24 Ascomycota, 8 mitosporic fungi, 1 Zygomycota, 1 Basidiomycota, 4 yeasts, 2 unidentified species and a sterile mycelium. Of these, 26 taxa occurred in the soil isolated from the human impacted lake shore, 5 from the ornithogenic site, 11 from the pristine site and 12 from the station site. The fungal strains were identified to genus or species level where possible using morphological characteristics and six selected strains were then sent to the National Taiwan Ocean University for molecular identification (Table 3.1).

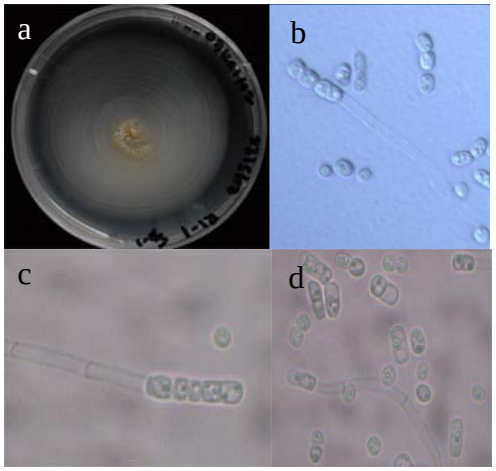
Table 3.1: Morphological identification of selected fungal strains found to be significant producers of extracellular hydrolytic enzymes

No	Micrograph	Morphological Description	Taxonomy
1.	*Geomyces pannorum*  	Incubation at different temperatures gave different colony colours. This varied across brown, grey and greenish brown at 25°C. Reverse pigmentation may include dark brown, maroon, dark green and white. Brown liquid observed on the colony. Fragile and powdery, concentric ring visible. At 4°C the colony was white when young, after approximately after 1 month changed to light brown. Main hyphae branch and sub branch to produce conidia, which were hyaline, had a rough surface, globose to subglobose, and measure 1.6-2.7µm Hyphae branched, septate; young mycelia were curled, clamp connection observed, sometimes conidiophores observed directly with hyphae producing spores without branching.	Phylum: Ascomycota Subphylum: Pezizomycotina Class: Leotiomyces Order: Helotiales Family: Myxotrichaceae Genus: Geomyces Species: *Geomyces pannorum* Identity confirmed by molecular analysis. Classified as a psychrotolerant species. Refer to Table 3.2

	 Figure 1a: Colony at 25°C; b: Colony at 4°C; Figure 1c-h: Light micrographs of conidia at 4°C, x40 magnification		
2.	Morphologically close to genus *Geomyces* (Ascomycete sp.17)  Figure 2a: Colony, b-d: Light micrographs of conidia of Ascomycete sp.17, x40 magnification	Colony greyish green, powdery, floccose, pink liquid observed on the colony, which diffused throughout the media, giving it a bright pink colour Hyphae were branched, with a rough surface, septate; spores produced at the end of sub-branching conidiogenous hyphae. Conidia subglobose to ellipsoid, with rough cell wall surface, size 1.6-2.8µm	Classified as a psychrophilic taxon based on growth only at 4°C in the current study

3.	Mitosporic fungi sp.18  Figure 3a: Colony of Mitosporic fungi sp. 18, b-d: light micrographs of Mitosporic fungi sp. 18, x20 magnification	Colony colour olivaceous green and white in bands, with a flat but not smooth surface, reverse pigmentation grey in the centre surrounded by white and olivaceous green, in fine concentric rings, and white at the margin; glossy structure, cream in colour, observed in the colony centre (similar to yeast) Hyphae hyaline, septate and branched Conidia produced at the phialide; phialides were lanceolate, hyaline, branching and sub-branching from the main hyphae and producing conidia. Some globose conidia produced as a cluster, others as individual conidia, measuring 1.03-1.5µm, and each containing one oil drop	Classified as psychrophilic taxon based on growth only at 4°C in the current study
4.	*Mrakia frigida/Mrakia nivalis*  	Colony white, showing concentric rings; glossy structure observed in the centre of the colony; slow growth, sour smell, colony contained mucus and had soft texture, no reverse pigmentation observed. Hyphae branched, hyaline, septate, spores bud directly from hyphae Teleospores observed.	Identity confirmed by molecular analysis Classified as psychrophilic species based on growth only at 4°C in the current study

	 Figure 4a: Colony of *Mrakia frigida*, b: teleospores of *Mrakia frigida* c-d: light micrographs of conidia of *Mrakia frigida*, x40 magnification	Conidia had a variety of shapes (elongated, oval, subglobose) and measured 2.2-6.5µm in length	
5	*Aureobasidium* sp.  Figure 5a: Colony of *Aureobasidium* sp.; b-d: light micrographs of conidia of *Au-*	Colony green, and white at the margin, flat surface, concentric rings present, no reverse pigmentation observed. Hyphae hyaline, septate and branched. Conidia hyaline, with smooth surface, oval shaped; conidia attached to basidia and some directly from the hyphae (without basidia), size 1.7-2.6 µm	Classified as psychrophilic taxa based on growth only at 4°C in the current study.

	reobasidium sp., magnification x20 (b) and x40 (c and d)		
6.	Yeast sp.16  Figure 6a: Colony of Yeast sp. 16; b-d: light micrographs of conidia of Yeast sp. 16, under magnification x40	Colony white, concentric rings present, with alternate rings raised; very smooth texture, glossy and glabrous, no reverse pigmentation Hyaline septate, branched and erect Conidia produced from conidiogenous hyphae, globose to square shaped. Measured1.5-2.6 µm in length	Classified as psychrophilic species based on growth only at 4°C in the current study.

3.1.2 Molecular identification

Six isolates showing the most significant activity for enzyme screening were subject to molecular identification (Table 3.2). The closest sequence matches for the ITS regions of the rRNA gene of *Geomyces* sp. 1 (AK07KGI1001R1-2), Deuteromycete sp. 25 (AK07KGI102R203), *Geomyces* sp. 2 (AK07KGI102R1-4), *Geomyces* sp. 2 (AK07KGI1001R2-1) and *Geomyces* sp. 1 (AK07KGI301R3-3) included a number of *Geomyces* spp., and their subsequent closest sequence matches with full species identity were the same, with 98-99% similarity to *Geomyces pannorum* (DQ189229, DQ189228, DQ189224). For *Mrakia* sp. (AK07KGI103 R2-1), the closest sequence matches were many unidentified species and *Mrakia* spp., while the closest sequence matches with full species identity were *Mrakia nivalis* (AF144484) and *M. frigida* (AF144482), both with 98% similarity. Sequences obtained have been deposited in Genbank, accession numbers JF720026-31.

Table 3.2: Fungal strains identified using molecular techniques

No	Taxon	Strain	GenBank accession number	Percentage of Similarity (%)
1	*Geomyces pannorum*	AK07KGI2001 R2-1(1) Station site	JF720030	98-99
2	*Geomyces pannorum*	AK07KGI102 R1-4(5) Human impacted lake shore	JF720028	98-99
3	*Geomyces pannorum*	AK07KGI301 R3-3(2) Ornithogenic site	JF720031	98-99
4	*Mrakia frigida*	AK07KGI103 R2-1(2) Human impacted lake shore	JF720029	99
5	*Geomyces pannorum*	AK07KGI1001 R1-2(1) Pristine site	JF720026	98-99
6	*Geomyces pannorum*	AK07KGI102 R2-3(1) Station site	JF720027	98-99

3.1.3 Frequency of soil microfungi

Table 3.3 illustrates the frequency of occurrence of the different taxa obtained at each sampling location in culture at 25°C. The most common species obtained in culture at 25°C was *Verticillium* sp., with frequency of occurrence of 60%. *Verticillium* sp. occurred most frequently in both the human impacted lake shore and station site soils. The most common taxa obtained from the ornithogenic site was *Mortierella* sp. *Geomyces pannorum* was the most common species obtained from the pristine site. The largest fungal diversity obtained in culture at this temperature was obtained from the human impacted lake shore.

Table 3.4 illustrates the frequency of occurrence of the different taxa obtained at each sampling location in culture at 4°C. *G. pannorum* recorded the highest frequency of occurrence, 63.3%. Again, the human impacted lake shore recorded the highest diversity of fungal taxa, followed by the pristine site, and the station and ornithogenic sites which shared the same diversity. *G. pannorum* was the most common fungus in the human impacted sites. In the ornithogenic site, Ascomycete sp. 17 and, again, *G. pannorum* occurred most frequently. Five taxa shared the same number of records, and were the most frequently-encountered fungi *G. pannorum*, Ascomycete sp.17, *Antarctomyces* sp.1, *Thelebolus* sp. and *Antarctomyces* sp.9 from pristine site.

The most common taxa recorded in this study regardless of their thermal classification (Table 3.5) was *Verticillium* sp., with a frequency of occurrence of 60%, followed by *G. pannorum* and *Mortierella* sp. with frequencies of 47% and 30%, respectively. *Phialophora* sp.5, Ascomycete sp.17, *Phialophora* sp.4, *Trichocladium* sp.36, Mitosporic fungi sp.18, *Penicillium* sp.1, *Trichocladium* sp.35, *Trichocladium* sp.37, *Antarctomyces* sp.1,

Trichocladium sp.6 classified as common species. The remaining taxa all classified as uncommon, with frequencies < 10%.

3.1.4 Diversity indices

Diversity indices calculated for each of the study sites are summarized in the Table 3.6. According to the Margalef Diversity Index, the human impacted lake shore had a higher diversity in comparison with the other sites, followed by the pristine site, the station site and the ornithogenic site.

Examination of Shannon Wiener Index values suggested that diversity and evenness of the species obtained differed between the collection sites. The human impacted lake shore was again the most diverse site, with the pristine and station sites being similar, and the ornithogenic site again being least diverse. In terms of evenness, the pristine site had the most even distribution of fungal taxa with the ornithogenic and station sites being similar, and the human impacted lake shore having the lowest evenness value.

3.1.5 Thermal classification

The 41 taxa identified included 16 mesophilic, four psychrotolerant and 21 psychrophilic taxa. The mesophiles included 12 ascomycetes and four mitosporic fungi, psychrotolerant fungi included three ascomycetes and one zygomycete, and psychrophilic fungi included nine ascomycetes, four mitosporic fungi, four yeasts, one basidiomycete, one sterile mycelium and two unidentified taxa. A majority of the taxa obtained were therefore classified as psychrophilic (Figure 3.1).

Table 3.3: Frequency of occurrence of soil microfungi in culture on agar plates at 25°C.

	Occurrence					
Taxa	**Human im-pacted lake shore**	**Ornitho nitho-genic site**	**Pristine site**	**Station site**	**Total**	**Frequency (% of plates)**
Verticillium sp.	13	0	0	5	18	**60.0**
Geomyces pannorum	6	0	3	0	9	**30.0**
Mortierella sp.	4	3	0	0	7	**23.3**
Phialophora Sp.5	6	0	1	0	7	**23.3**
Penicillium Sp.1	1	0	0	5	6	**20.0**
Phialophora Sp.4	5	0	0	0	5	**16.7**
Trichocladium Sp.6	1	0	0	4	5	**16.7**
Trichocladium sp.36	0	0	0	4	4	**13.3**
Trichocladium sp.35	0	0	0	3	3	**10.0**
Trichocladium sp. 37	0	0	0	3	3	**10.0**
Phialophora Sp.10	2	0	0	0	2	**6.7**
Mitosporic fungi Sp.14	1	1	0	0	2	**6.7**
Penicillium sp.2	0	0	0	2	2	**6.7**
Phialophora Sp.7	1	0	0	0	1	**3.3**
Mitosporic fungi Sp.8	1	0	0	0	1	**3.3**
Mitosporic fungi sp.9	0	0	0	0	1	**3.3**
Mitosporic fungi Sp.11	1	0	0	0	1	**3.3**
Ascomycete Sp.12	1	0	0	0	1	**3.3**
Mitosporic fungi Sp.13	1	0	0	0	1	**3.3**
Trichocladium sp.38	0	0	0	1	1	**3.3**
Fusarium sp.	0	0	0	1	1	**3.3**
Total	**44**	**4**	**4**	**28**	**81**	

Table 3.4: Frequency of occurrence of soil microfungi in culture on agar plates at 4°C

	Occurrence					
Taxa	**Human impacted lake shore**	**Ornitho nitho-genic site**	**Pristine site**	**Station site**	**Total**	**Frequency (% of plates)**
Geomyces pannorum	10	3	2	4	19	**63.3**
Mortierella sp.	8	2	0	1	11	**36.7**
Ascomycete sp.17	2	3	2	0	7	**23.3**
Mitosporic fungi sp. 18	4	0	0	0	4	**13.3**
Antarctomyces sp.1	1	0	2	0	3	**10.0**
Aureobasidium sp.	2	0	0	0	2	**6.7**
Yeast sp.16	2	0	0	0	2	**6.7**
Mrakia frigida	2	0	0	0	2	**6.7**
Thelebolus sp.	0	0	2	0	2	**6.7**
Antarctomyces sp.9	0	0	2	0	2	**6.7**
Penicillium sp.1	0	0	0	2	2	**6.7**
Yeast sp.24	1	0	0	1	2	**6.7**
Antarctomyces sp.2	1	0	0	0	1	**3.3**
Antarctomyces sp.4	1	0	0	0	1	**3.3**
Antarctomyces sp.6	0	1	0	0	1	**3.3**
Yeast sp.23	1	0	0	0	1	**3.3**
Mitosporic fungi sp. 29	1	0	0	0	1	**3.3**
Antarctomyces sp.8	0	0	1	0	1	**3.3**
Unidentified sp.2	0	0	1	0	1	**3.3**
Unidentified sp.3	0	0	1	0	1	**3.3**
Ascomycete sp.1	0	0	1	0	1	**3.3**
Sterile mycelia	0	0	1	0	1	**3.3**
Trichocladium sp.6	0	0	0	1	1	**3.3**
Mitosporic fungi sp.28	1	0	0	0	1	**3.3**
Total	**37**	**9**	**15**	**9**	**70**	

Table 3.5: Overall frequency of occurrence of soil microfungi

Taxa	**Total**	**Overall frequency of occurrence (% of plates)**
Verticillium sp.	18	60.00
Geomyces pannorum	28	46.67
Mortierella sp.	18	30.00
Phialopora sp.5	7	23.33
Ascomycete sp.17	7	23.33
Phialophora sp.4	5	16.67
Trichocladium sp.36	4	13.33
Mitosporic fungi sp. 18	4	13.33
Penicillium sp.1	8	13.33
Trichocladium sp.35	3	10.00
Trichocladium sp. 37	3	10.00
Antarctomyces sp.1	3	10.00
Trichocladium sp.6	6	10.00
Phialophora sp.10	2	6.67
Mitosporic fungi sp.14	2	6.67
Penicillium sp.2	2	6.67
Aureobasidium sp.	2	6.67
Yeast sp.16	2	6.67
Mrakia frigida	2	6.67
Thelebolus sp.	2	6.67
Antarctomyces sp.9	2	6.67
Yeast sp.24	2	6.67
Mitosporic fungi sp.28	1	3.33
Phialophora sp.7	1	3.33
Mitosporic fungi sp.8	1	3.33
Mitosporic fungi sp.9	1	3.33
Mitosporic fungi sp.11	1	3.33
Ascomycete sp.12	1	3.33
Mitosporic fungi sp.13	1	3.33
Trichocladium sp.38	1	3.33
Fusarium sp.	1	3.33
Antarctomyces sp.2	1	3.33
Antarctomyces sp.4	1	3.33
Antarctomyces sp.6	1	3.33
Yeast sp. 23	1	3.33
Mitosporic fungi sp. 29	1	3.33
Antarctomyces sp.8	1	3.33
Unidentified sp.2	1	3.33
Unidentified sp.3	1	3.33
Ascomycete sp.1	1	3.33
Sterile mycelia	1	3.33
Total occurrence	151	

Table 3.6: Diversity indices of soil microfungi from Fildes Peninsula

Index	Human impacted lake shore	Ornitho-nitho-genic site	Pristine site	Station site
Margalef Diversity Index	5.63	1.85	3.40	3.09
Shannon Wiener Index	2.7	1.56	2.29	2.14
Species Evenness	0.83	0.87	0.95	0.86

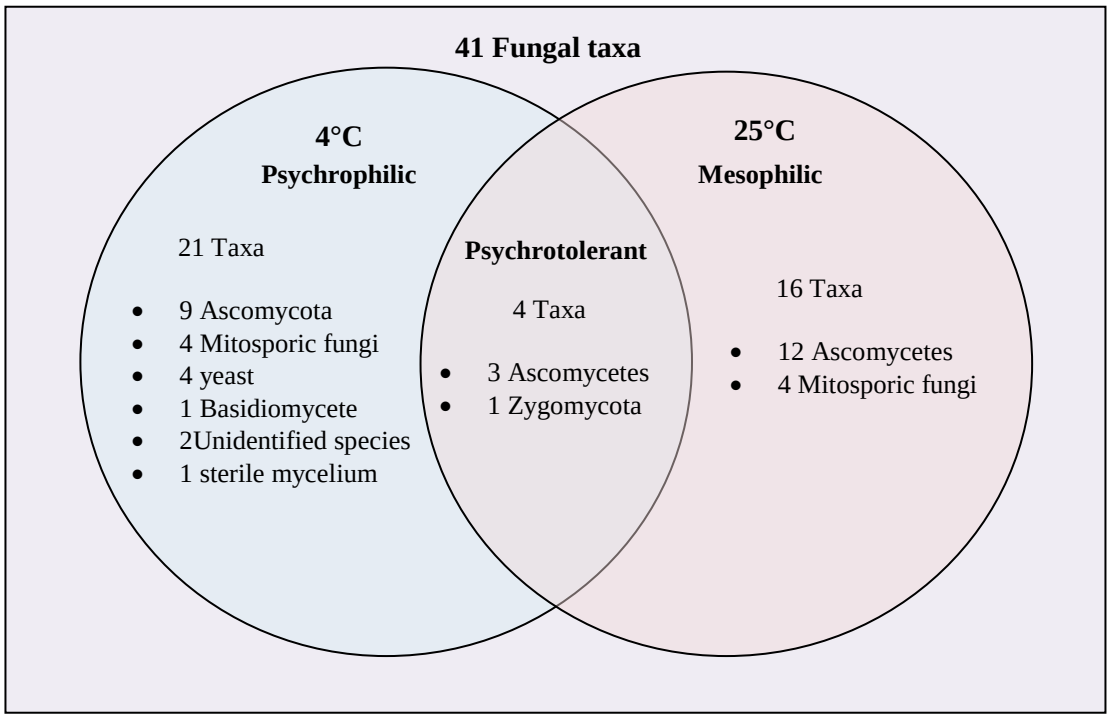


Figure 3.1: Thermal classification of soil microfungi from King George Island

3.2 Enzyme screening of psychrophilic and psychrotolerant soil microfungi

A total of 28 psychrophilic and psychrotolerant isolates were selected from the 41 fungal taxa isolated, and screened for their hydrolase (amylase, cellulose and protease) enzyme activities. These 28 strains were selected since we were only interested in psychrophilic and psychrotolerant fungi. Therefore, the balance of 13 mesophilic fungi were not tested of their enzyme production.

3.2.1 Amylase

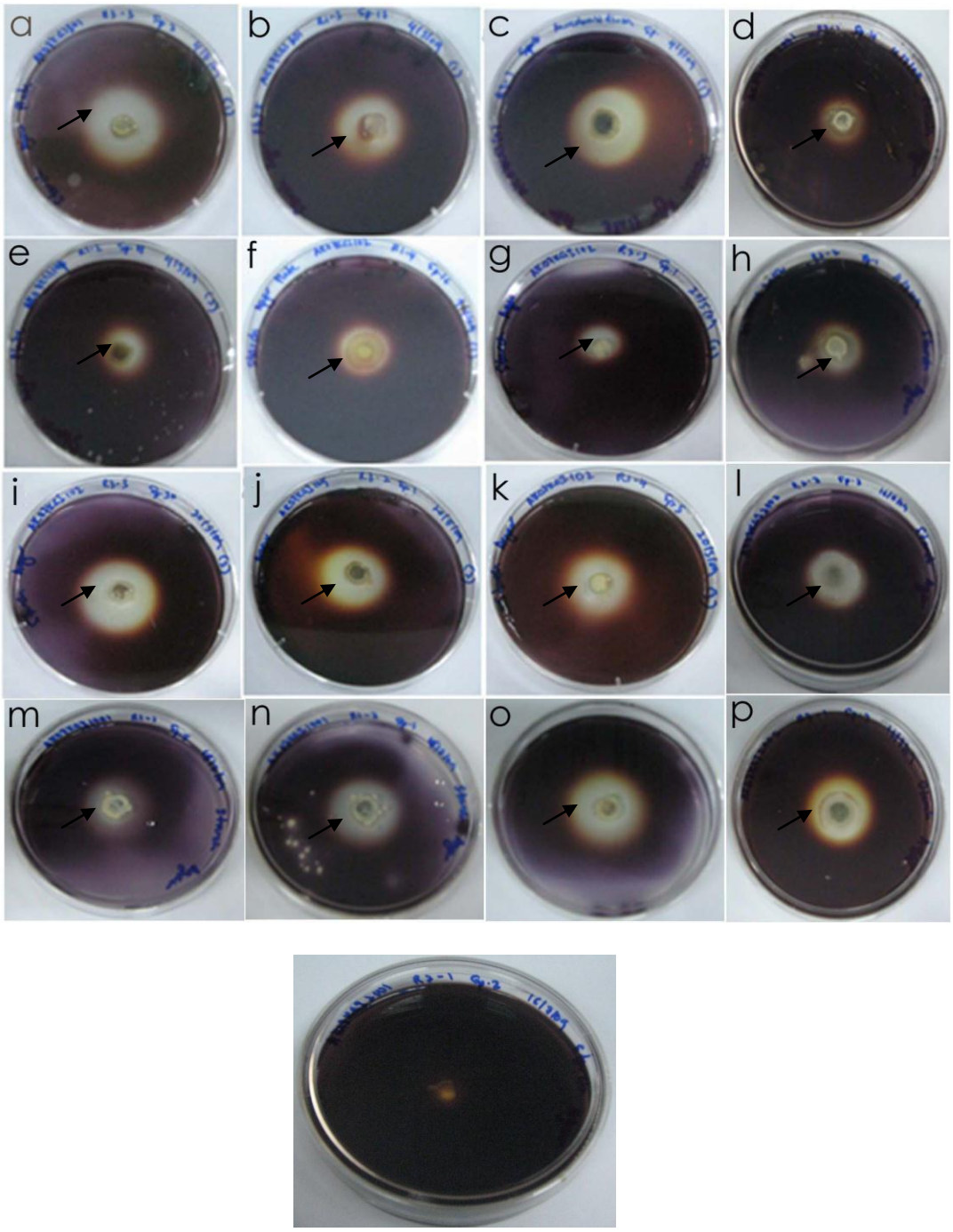
Sixteen of the 28 strains showed positive amylase activity (Figure 3.2). A range of different strains of *G. pannorum* had showed significant activity for amylase. *G. pannorum* (strain AK07KGI2001 R2-1) recorded the highest Relative Activity (RA) for amylase, of 2.14. The relative amylase activities of the different strains are summarized in Figure 3.3.

3.2.2 Cellulase

Twenty-three strains showed cellulase activity (Figure 3.4). *M. frigida* was the highest cellulase producing strain with RA value 1.70. The relative cellulase activities are illustrated in Figure 3.5.

3.2.3 Protease

Twenty-one strains showed positive protease activity (Figure 3.6). The species with highest protease production was *G. pannorum* (strain AK07KGI102 R2-3), with an RA value of 2.48. The relative protease activities are illustrated in Figure 3.7.



Control

Figure 3.2: Plates with positive activity for amylase (arrows showing activity zones) a: *Geomyces pannorum* b: Ascomycete sp. 17 c: *Aureobasidium* sp. d: *Geomyces pannorum* e: Mitosporic fungi sp. 18 f: Yeast sp. 16 g: *Geomyces pannorum* h: *Geomyces pannorum* i: *Geomyces pannorum* j: *Geomyces pannorum* k: *Geomyces pannorum* l: *Trichocladium* sp. 6 m: *Geomyces pannorum* n: *Geomyces pannorum* o: *Geomyces pannorum* p: *Penicillum* sp.

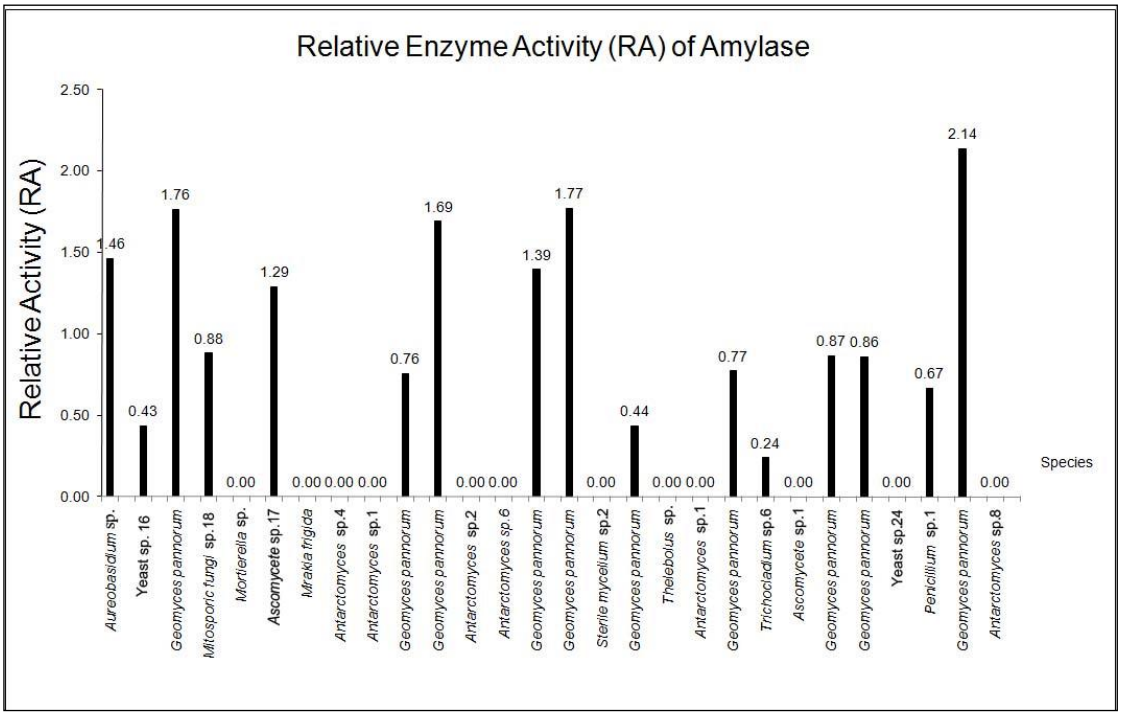
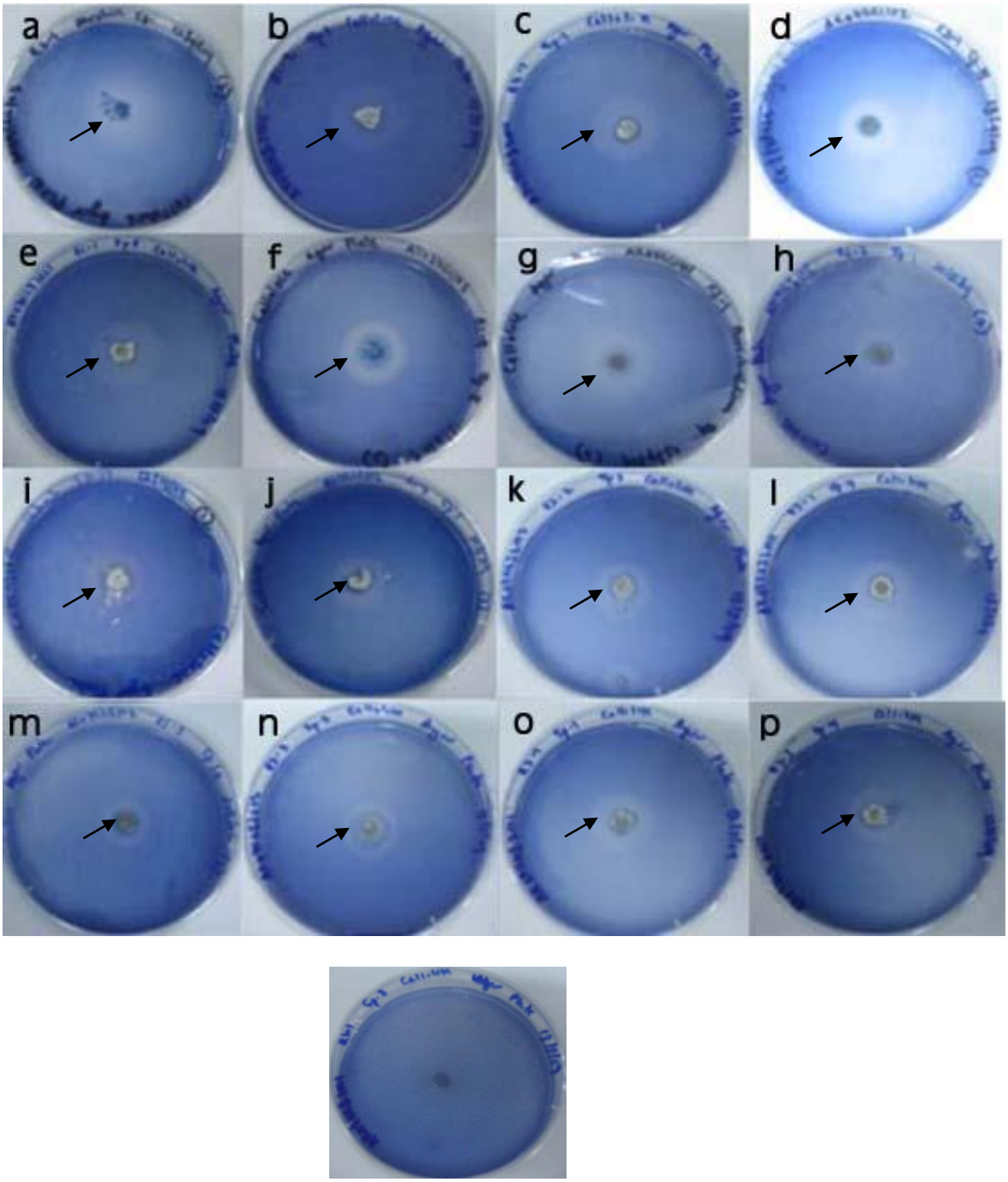


Figure 3.3: Relative activity for amylase on agar plates



Control

Figure 3.4: Plates with cellulase activity (arrows showing activity zones) a: *Mrakia frigida* b: *Geomyces pannorum* c: *Geomyces pannorum* d: *Geomyces pannorum* e: Mitosporic fungi sp. 18 f: Yeast sp. 16 g: *Aureobasidium* sp. h: *Geomyces pannorum* i: Ascomycete sp. 17 j: *Geomyces pannorum* k: *Geomyces pannorum* l:*Geomyces pannorum* m: *Geomyces pannorum* n: *Penicillium* sp. o: *Geomyces pannorum* p: *Geomyces pannorum*

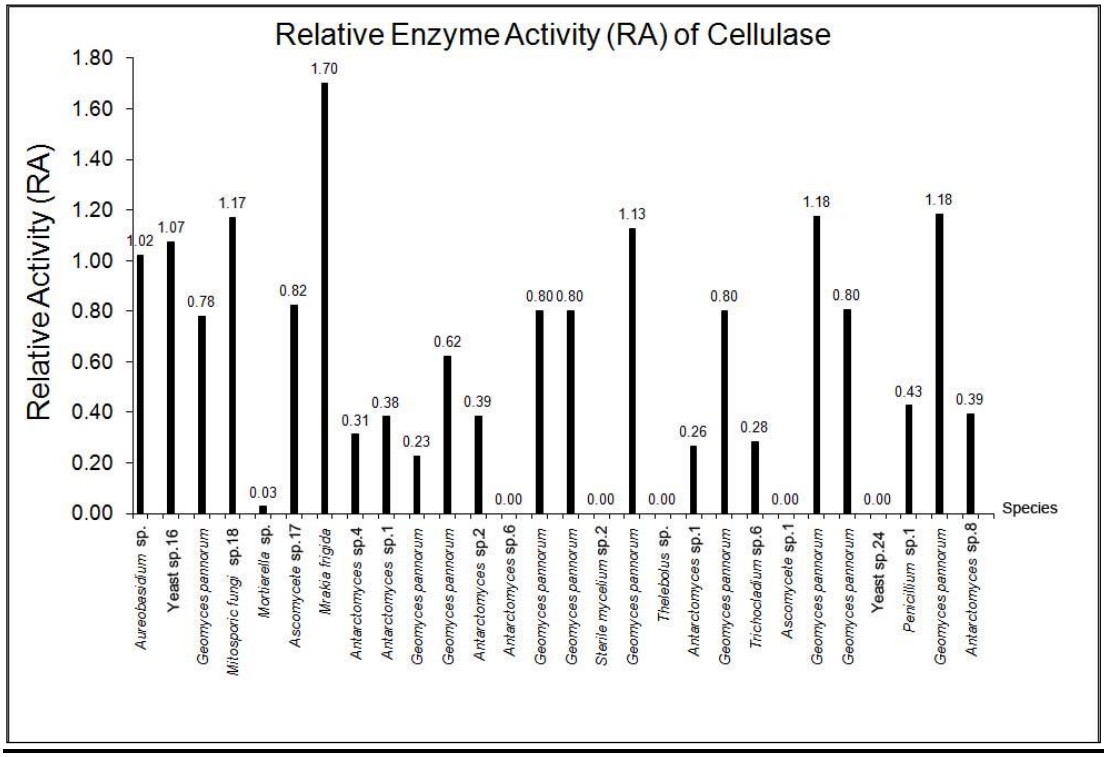
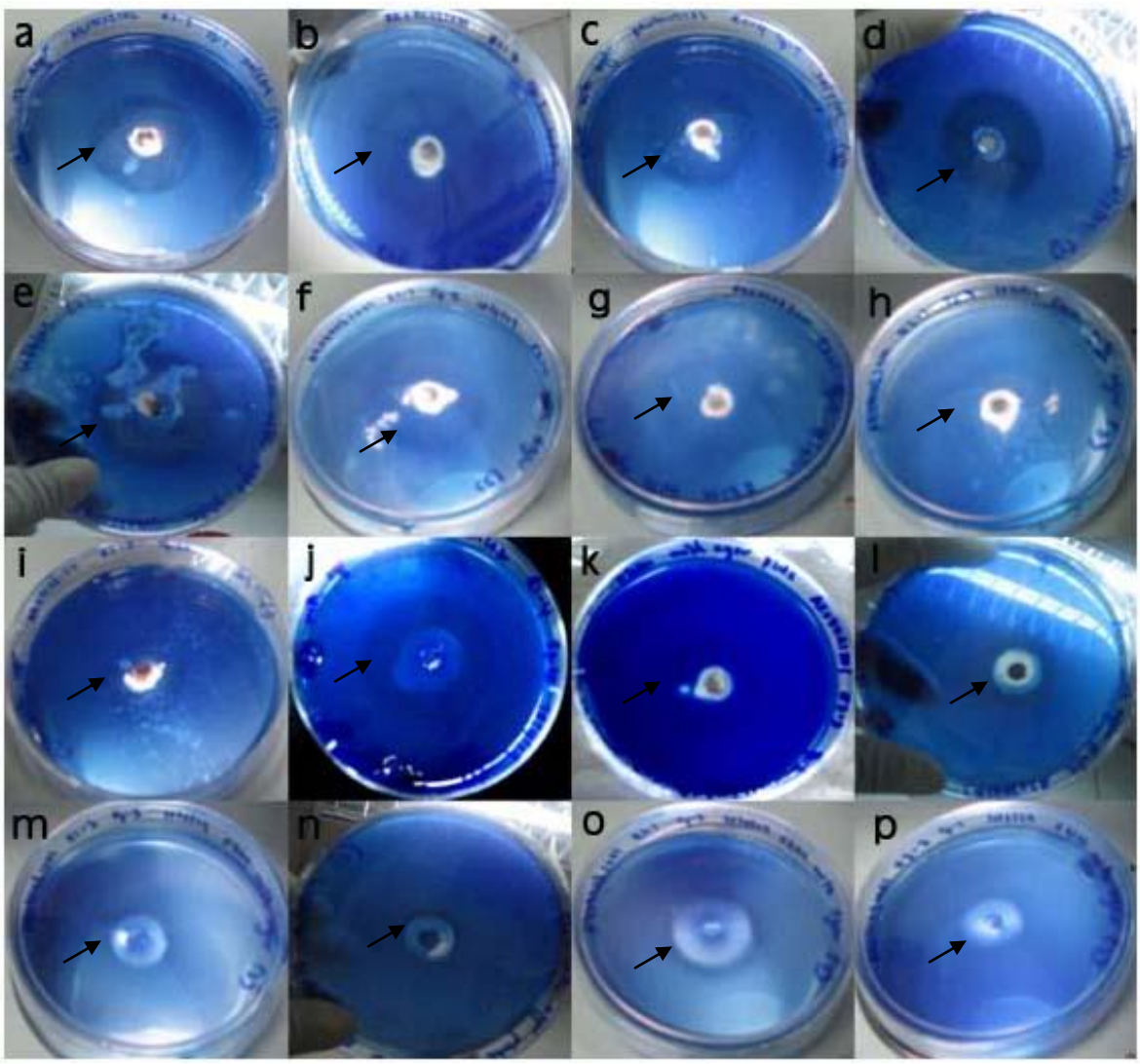
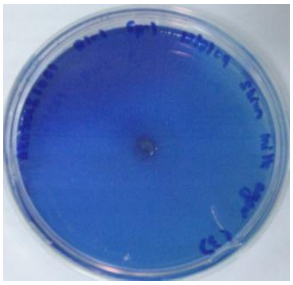


Figure 3.5: Relative activity for cellulase on agar plates





Control

Figure 3.6: Plates with positive activity for protease (arrows showing activity zones)a: *Geomyces pannorum* b: *Geomyces pannorum* c: *Geomyces pannorum* d: *Mrakia frigida* e: Mitosporic fungi sp. 17 f: *Geomyces pannorum* g: *Geomyces pannorum* h: *Geomyces pannorum* i: *Geomyces pannorum* j: Yeast sp. 16 k: *Geomyces pannorum* l: *Aureobasidium* sp. m: Sterile mycelium sp. 2 n: Mitosporic fungi sp. 18 o: Ascomycete sp. 1 p: *Thelebolus* sp.

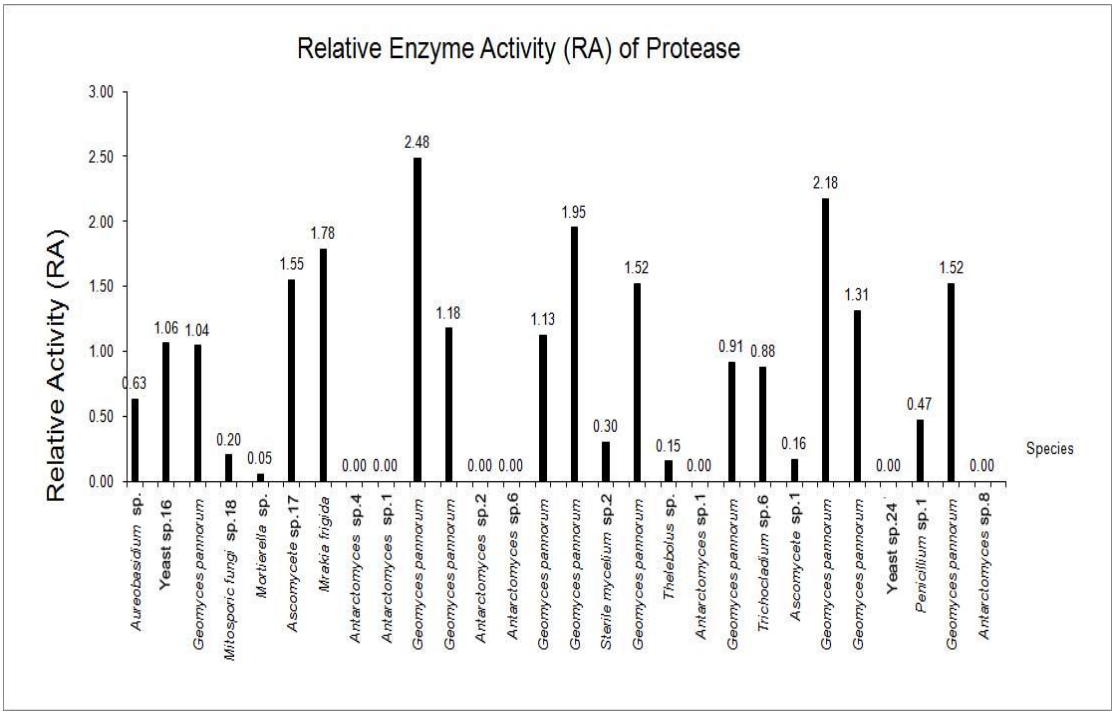


Figure 3.7: Relative Activity for protease on agar plates

The enzyme activities above are summarized in the Table 3.7. The strongest activities were shown by *G. pannorum* for amylase and protease and *M. frigida* for cellulase (Figure 3.8). However, there was no clear indication of any association between enzyme production and the different sampling sites.

Table 3.7: Relative enzyme activities (RA) of the fungal strains for extracellular amylase, cellulase and protease production. A, C and P represent amylase, cellulase and protease respectively

Species	Isolate number	Human impacted lake shore			Ornithogenic site			Pristine site			Station site		
		A	**C**	**P**	**A**	**C**	**P**	**A**	**C**	**P**	**A**	**C**	**P**
Mortierella sp.	AK07KGI105 R3-1	0.0	0.0	0.1									
Aureobasidium sp.	AK07KGI101 R3-1(2)	1.5	1.0	0.6									
Yeast sp.16	AK07KGI102 R1-4(2)	0.4	1.1	1.1									
Antarctomyces sp.1	AK07KGI102 R1-3(2)	0.0	0.4	0.0									
Antarctomyces sp.1	AK07KGI1001 R1-1(1)							0.0	0.3	0.0			
Antarctomyces sp.2	AK07KGI102 R2-3(5)	0.0	0.4	0.0									
Antarctomyces sp.4	AK07KGI102 R3-2(5)	0.0	0.3	0.0									
Antarctomyces sp.6	AK07KGI301 R2-2				0.0	0.0	0.0						
Ascomycete sp.17	AK07KGI105 R3-2(6)	1.3	0.8	1.6									
Mitosporic fungi sp. 18	AK07KGI102 R2-1(2)	0.9	1.2	0.2									
Mrakia frigida	AK07KGI103 R2-1(2)	0.0	1.7	1.8									
Geomyces pannorum	AK07KGI102 R2-3(1)	0.8	0.2	2.5									
Geomyces pannorum	AK07KGI102 R1-4(5)	1.8	0.8	2.0									
Geomyces pannorum	AK07KGI301 R3-3(2)				1.8	0.8	1.0						
Geomyces pannorum	AK07KGI1001 R1-1(5)							0.4	1.1	1.5			
Geomyces pannorum	AK07KGI1001 R1-2(1)							0.9	1.2	2.2			
Geomyces pannorum	AK07KGI2001 R2-1(1)										2.1	1.2	1.5
Geomyces pannorum	AK07KGI2001 R3-1										0.9	0.8	1.3

Geomyces pannorum	AK07KGI2002 R2-2(1)								0.8	0.8	0.9
Geomyces pannorum	AK07KGI102 R3-3	1.7	0.6	1.2							
Thelebolus sp.	AK07KGI1001 R3-2(1)					0.0	0.0	0.2			
Antarctomyces sp.8	AK07KGI1001 R1-1(4)					0.0	0.4	0.0			
Ascomycete sp.1	AK07KGI1001 R1-1(2)					0.0	0.0	0.2			
Sterile mycelia sp.2	AK07KGI1001 R1-2(2)					0.0	0.0	0.3			
Trichocladium sp.6	AK07KGI2002 R2-2(2)								0.2	0.3	0.9
Penicillium sp.1	AK07KGI2002 R3-1(2)								0.7	0.4	0.5
Yeast sp.24	AK07KGI2001 R2-1(2)								0.0	0.0	0.0
Geomyces pannorum	AK07KGI105 R3-2(1)	1.4	0.8	1.1							

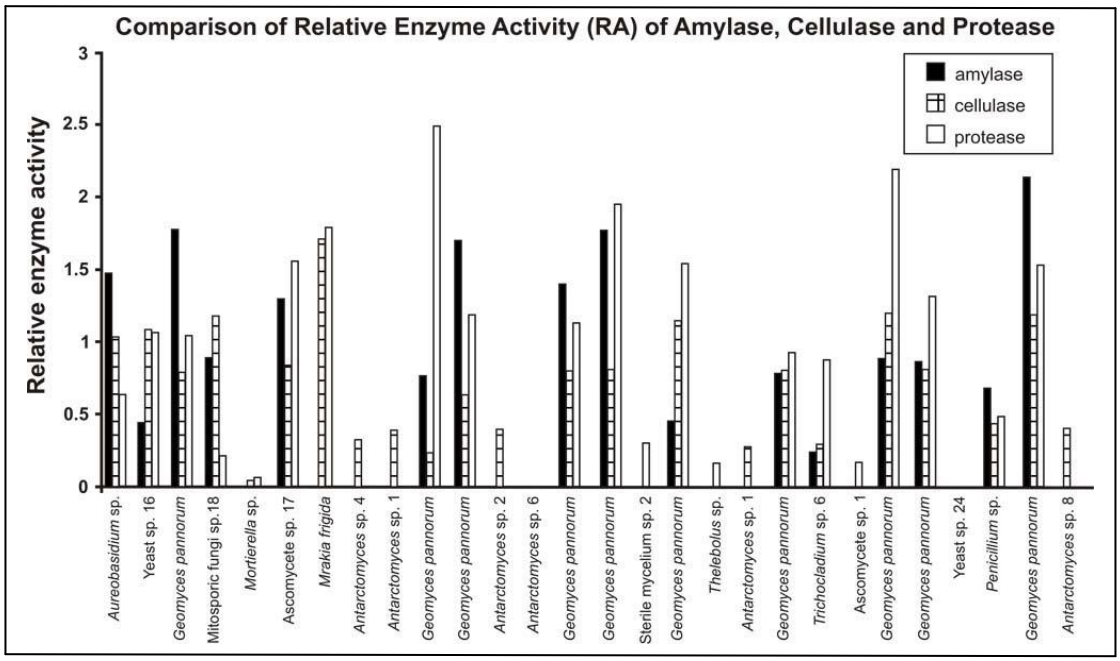


Figure 3.8: Comparison of relative enzyme activity of amylase, cellulase and protease across the 28 strains examined

3.3 Enzyme assay

3.3.1 Amylase production of selected fungal strains

The three strains showing highest RA for amylase were selected for this assay; all three were different strains of *G. pannorum*. These three strains were isolated respectively from the human impacted lake shore, ornithogenic and station sites.

3.3.1.1 Total protein content

In order to determine total protein content in the cell-free culture, a standard curve was constructed using Bovine Serum Albumin (BSA) (Figure 2.3). This standard curve was then used to determine the total protein content in the three *G. pannorum* strains.

Based on the standard curve constructed, total protein contents in the cell-free supernatant obtained from the selected strains were determined (Table 3.8). The highest concentration was quantified from the *G. pannorum* strain which was isolated from the station site, although all three strains gave similar values.

Table 3.8: Total protein content of the cell free supernatant liquids in crude amylase

Strain	**Total Protein Content (mg/ml)**
Geomyces pannorum (station site - 2001 R2-1 sp.1)	0.021
Geomyces pannorum (human impacted lake shore - 102 R1-4 sp.5)	0.019
Geomyces pannorum (ornithogenic site - 301 R3-3)	0.018

3.3.1.2 Amylase activity

Amylase activity was determined based on the reducing sugar method of Miller (1959) and Ghose (1987). The liberated glucose concentration was calculated based on a standard curve which was constructed using glucose (Figure 3.9).

The glucose production and specific activity of the three strains are given in Table 3.9. The strain obtained from the ornithogenic site had the highest glucose production and enzyme activity, of 0.058 µmol/min/ml and 3.208 U/mg, respectively. The next strongest amylase producing strain was isolated from the human impacted lake shore. The strain obtained from the station site showed the weakest activity.

3.3.2 Cellulase production of selected fungal strains

The three strains showing the highest RA for cellulase were selected for measurement of enzyme activity. These strains included *M. frigida* isolated from the human impacted lake shore, which had the highest RA for cellulose, followed by two strains of *G. pannorum* isolated from the pristine site and the station site.

3.3.2.1 Total protein content

A standard curve was constructed using BSA (Figure 2.4) to calculate total protein contents of the three strains. The total protein contents of the three strains are given in Table 3.10.

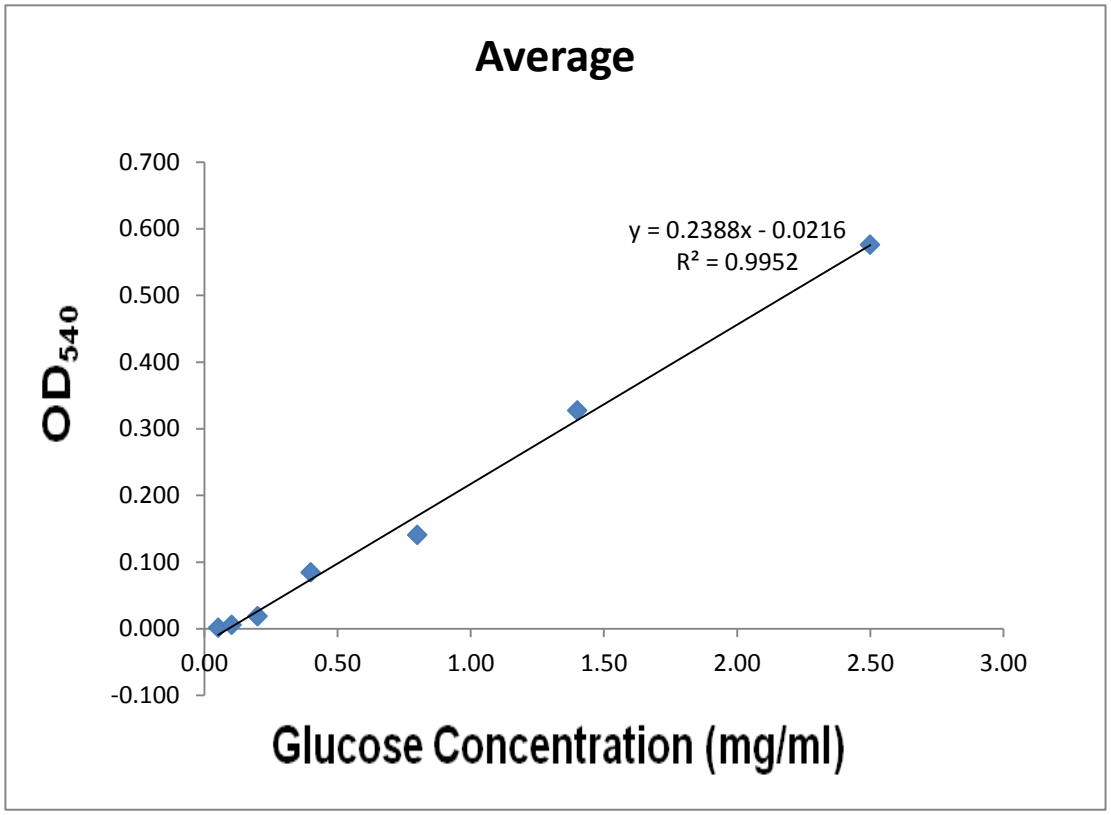


Figure 3.9: Standard curve using glucose as a standard for amylase

Table 3.9: Glucose production and the specific activity of the amylase enzyme

Strain	**Glucose production (µmol/min/ml)**	**Specific activity (U/mg)**
Geomyces pannorum (station site - AK2001 R2-1)	0.025	1.209
Geomyces pannorum (human impacted lake shore - AK102 R1-4)	0.047	2.466
Geomyces pannorum (ornithogenic site- AK301 R3-3)	0.058	3.208

Table 3.10: Total protein content of the cell free culture in crude cellulase

Strain	**Total Protein Concentration (mg/ml)**
Mrakia nivalis/Mrakia frigida (human impacted lake shore - AK07KGI103 R2-1)	0.048
Geomyces pannorum (pristine site - AK07KGI1001 R1-2)	0.059
Geomyces pannorum (station site - AK07KGI2001 R2-1)	0.055

3.3.2.2 Cellulase activity

Cellulase activity was calculated based on the reducing sugar assay of Miller (1959) and Ghose (1987), using a glucose standard curve (Figure 3.10).

Cellulase activity (glucose production) was marginally greatest in the *G. pannorum* strain isolated from the pristine site (Table 3.10), although the *G. pannorum* strain isolated from the human impacted lake shore had higher specific activity.

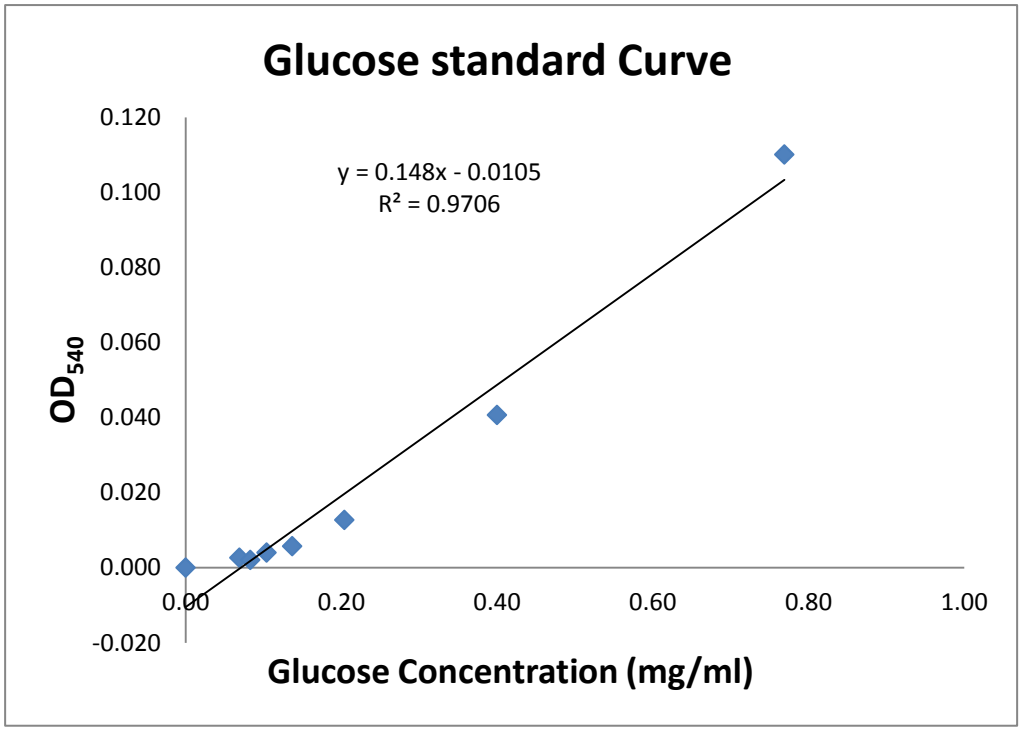


Figure 3.10: Standard curve using glucose as a standard for cellulase

Table 3.11: Glucose production and the specific activity of cellulase

Strain	**Glucose production (µmol/min/ml)**	**Specific activity (U/mg)**
Geomyces pannorum (pristine site - AK1001 R1-2)	0.01255	0.213
*Mrakia nivalis/Mrakia frigid*a (human impacted lake shore - AK103 R2-1)	0.01206	0.251
Geomyces pannorum (station site - AK2001 R2-1)	0.01088	0.198

4.0 Discussion

4.1 Diversity of fungi from King George Island, Peninsula Antarctica

The total fungal diversity on the Earth has been estimated at 1.5M species (Hawksworth, 1991), although more recent lower estimates exist (e.g. 720,256 species; Schmit and Mueller, 2007). Hawksworth's (1991) estimate is still accepted widely, giving a reasonable ratio of fungal to plant diversity of 6:1. The Dictionary of Fungi (Kirk *et al.*, 2008) stated that described number of species was 97330. In the Antarctic region alone, more than 1000 species have been reported (Bridge *et al*., 2008b), only 1% of the total number of fungi described in the world, possibly indicating the strong environmental constrains in this region. Molecular techniques have provided rapid recent advances in the identification of fungi. However, morphological identification continues to give us an opportunity to understand and appreciate fungi and their role in the environment. In this study we used morphological techniques to identify fungi to genus level. However, confirmation of identity using molecular methods is an important additional tool for a more reliable identification (Arenz *et al*., 2006). Previous molecular analyses have suggested that maritime Antarctic soil hosts greater eukaryotic diversity compared to continental Antarctica (Lawley *et al*., 2004; Yergeau *et al.*, 2007). In this study we obtained molecular identification for six of the best-yielding (extracellular enzyme producing) isolates with potential value in future biotechnological development. However, there are also situations where DNA sequencing for identification is not always reliable - for instance sequences identified as cf. *Phoma* or *Phoma*-like coelomycetes have later been revealed to be anamorphs of *Pezicula* or *Neofabraea* species (Bridge *et al*., 2003).

Antarctica hosts very simple ecosystems, some with the lowest biodiversity on the globe (Wall, 2005). Nevertheless, King George Island is rich in diversity of fungi in comparison with other reports from this region. In this study 41 fungal strains (based on morphological grounds) were isolated from 10 soil samples. This is rich in comparison with previous studies from Windmill Island (22 genera from 1228 isolates) (Azmi and Seppelt, 1997), Victoria Land (18 genera from 120 isolates) (Tosi *et al*., 2002), and Taylor Valley (114 occurrence of fungi from 160 soil samples) (Connell *et al*., 2006). Other diversity studies conducted using molecular analyses cannot be compared easily with the present study (Connell *et al*., 2006; Bridge and Newsham, 2009; Duncan *et al*., 2010).

In the current study, 24 strains belonged to the ascomycetes, including 10 anamorphic ascomycetes. This result is consistent with the reports of Moller and Dreyfuss (1996), Ruisi *et al*. (2007) and Ludley and Robinson (2008). These studies reported a majority of species being anamorphic ascomycetes. These fungi adapt to the extreme environment of Antarctica by losing the ability for sexual reproduction and having a shorter lifecycle (Ruisi *et al*., 2007). Studies of Victoria Land soil also documented a similar result, identifying anamophic or teleomorphic ascomyctetes, zygomycetes and basidiomycetes as common fungi (Adams *et al*., 2006). There are also some exceptional cases where *Thelebolus* sp., which is a teleomorph ascomycete, has been frequently isolated, using reduction of its ascomata as an adaptation strategy (de Hoog *et al*., 2005).

Previous investigators have reported a greater proportion of yeasts compared to filamentous fungi in studies from continental Antarctica (Connell *et al*., 2008; Ludley and Robinson, 2008). This was supported by Arenz *et al*. (2006) who isolated basidiomycetous yeast but no filamentous basidiomycetes. The possible reason behind this could be because yeasts are well adapted to the extreme environment and they are able to thrive by utilizing simple sugars (Vishniac and Baharaeen, 1982). However, in the current study we docu-

mented more filamentous fungi than yeasts. This may relate to the maritime Antarctic having a milder climate in comparison with continental Antarctica. A considerable proportion of the fungi isolated from the Fildes Peninsula, King George Island were cosmopolitan. For instance, genera such as *Penicillium, Cadophora, Geomyces, Verticillium, Trichocladium, Mortierella and Phialophora* are all found elsewhere in the world. Adams *et al*. (2006), Ruisi *et al*. (2007) and Onofri *et al*. (2007a) found similar results in their respective studies. Bridge *et al*. (2008b) estimated that only 2-3% fungi are endemic to Antarctica. Fungi that have been introduced to Antarctica are probably most likely to be transported by air from the nearest continent of South America (Chalmers, 1996), and by vectors like birds and through anthropogenic actions (Bridge *et al*., 2008b; Arenz *et al*., 2010).

Among the five sampling sites we examined, the human impacted lake shore site harbored the largest number of fungi. Here, 66.7% of the overall diversity obtained was isolated at 25°C and 58.3% at 4°C (63.4% of diversity overall). At 25°C, the highest number of occurrences was recorded by *Verticillium* sp., with this being *G. pannorum* at 4°C. Other studies support the finding of highest fungal diversity being isolated from human impacted sites (Azmi and Seppelt, 1997). Generally, human impacted sites are prone to organic contamination, for instance through fuel spills, associated with vehicle and machinery use. Hydrocarbons in the soil can act as a substrate for the microfungi (Bossert and Bartha, 1984), as some can degrade hydrocarbons (Atlas and Cerniglia, 1995) and inhibit the growth of other microorgansisms (Hughes *et al.,* 2007). Aislabie *et al.* (2001) found an increased number of fungi from oil contaminated soil at Scott Base and Marble Point (continental Antarctica).

The lowest diversity of fungi (14.6% of overall diversity) was found at the ornithogenic site at both incubation temperatures. At 25°C, 9.5% of fungi were isolated and at 4°C it was 16.7%. The highest frequency of occurrence was recorded by *Mortierella* sp.

at 4°C and *G. pannorum* and Ascomycete sp.17 at 25°C. *Mortierella* sp. is a psychrotolerant species. Its association with ornithogenic soil may relate to the high Na^+, K^+, Cl^- and SO_4^{2-} content of such soils. This genus has been reported to have association with hydrocarbon degradation in cold environments (Hughes *et al.*, 2007) and is also reported from moss covered soil (Bridge and Newsham, 2009). This genus has been previously reported from South Georgia (Hurst, Pugh and Walton, 1983), Victoria Land (Adams *et al*., 2006; Gesheva, 2009), maritime Antarctica (Malosso *et al*., 2006) and Rothera Point (Hughes *et al*., 2007). Its low frequency of occurrence in the present study in the ornithogenic site may be as a result of low sampling effort in this area.

Our sampling of pristine sites generated 11 fungal taxa. These contributed 41.7% of fungi obtained at 4°C, and 9.5% at 25°C (26.8% of fungi overall). The highest frequency of occurrence was recorded by *G. pannorum* at 25°C. This is in line with previous reports of *G. pannorum* from both pristine and biotically influenced sites in the Antarctic region (Azmi and Seppelt, 1997). *G. pannorum*, Ascomycete sp. 17, Ascomycete sp. 1, *Thelebolus* sp. and *Antarctomyces* sp. 9 were found to be the most frequent fungi at 4°C. Mostly we isolated teleomorph ascomycetes (*Antarctomyces* sp.), which may indicate an ecological role in this region. Previously, Stchigel and Cormack (2003) reported only two teleomorph acomycetes (*Apiosordaria antarctica* Stchigel and Guarro 2003 and *Thielavia antarctica* Stchigel & Guarro 2003) from King George Island. They also stated that only a small number of fungi belonged to the ascomycete group. However this statement is not consistent with the present study reporting 16 anamorph ascomycetes and 8 teleomorph ascomycetes.

The station area, which is also a human impacted site, recorded the second greatest number of fungal species. We recovered 12 fungal taxa from this site, or 29.3% of overall

diversity. Of these, 11 were anamorphic ascomycetes and one was a yeast. At 25°C, the percentage was 42.9 and at 4°C it was 20.8%, proportions that are in line with Azmi and Seppelt (1997). Gesheva (2010) also reported an elevated occurrence of actinomycetes near to a research station.

Overall, at 25°C, the most common taxa isolated from King George Island were *Verticillium* sp. and *G. pannorum*, with frequencies of occurrence of 60% and 30%, respectively. Six taxa were categorized as common, including *Mortierella* sp., *Phialophora* sp.5, *Penicillium* sp.1, *Phialophora* sp.4, *Trichocladium* sp.6 and *Trichocladium* sp. 36. The remaining taxa were categorized as less common. At 4°C, two taxa were recorded as the most common, *G. pannorum* and *Mortierella* sp., with frequencies of occurrence 63.3% and 36.7%, respectively. Ascomycete sp. 17 and Mitosporic fungi sp. 18 were found to be common taxa, (23.3% and 13.3%, respectively), with the remainder being less common taxa. The three most common taxa, *Verticillium* sp., *G. pannorum* and *Mortierella* sp. may have an important ecological role in this extreme environment.

Representatives of the genus *Geomyces* have been frequently reported from the Ross Sea Region. The current study revealed a similar result, finding *G. pannorum* as the most common species (cf. Arenz *et al*., 2006). *G. pannorum* has previously been isolated from MacRobertson and Enderby Lands (Fletcher, 1985). The genus was previously known as *Chrysosporium*, and was dominant in oil free soils from the Ross Sea region (Aislabie *et al*., 2001). According to Marshall (1998) *G. pannorum* is a keratinophilic fungus. Keratinophilic fungi are capable of degrading keratin from bird feathers, which may explain why this species is found abundantly in penguin rookeries and other ornithogenic sites (Marshall, 1998). Such a relationship is not clear from the current study, with the species being found almost at all the sites we sampled, although King George Island is overall a biotically rich

environment in comparison with the more extreme locations in Victoria Land. This is probably because it has a major role in nutrient cycles in Antarctica (Arenz *et al*., 2006).

This species also been isolated from pristine sites (Kerry, 1990a) and sites with limited biotic impacts (Azmi and Seppelt, 1997). *G. pannorum* was also reported to be frequently isolated from Ross Island historic huts (Arenz and Blanchette, 2011; Blanchette *et al*., 2010), Bunger Hills (Barker, 1977), Livingston Island (Tosi *et al*., 2010) and Vestfold Hills (Rounsevell, 1981). This statement is consistent with Arenz, Held and Jurgens (2009) and Vishniac (1996), who reported *G. pannorum* to occur frequently in respective studies. Arenz and Blanchette (2011) also reported this genus was the most abundant in the Antarctic Peninsula. *G. pannorum* recorded one of the highest frequencies of occurrence in the current study, with Ascomycete sp.17 (which is taxonomically close to *Geomyces*) also being frequently encountered. Even though this species is recorded widely in Antarctica, it has reduced abundance near oil contaminated sites (Kerry, 1990b).

We recorded *Penicillium* sp. in the current study, but as one of the less common species. It has also been reported from other parts of Antarctica such as Ross Island (Arenz *et al*., 2010), the Palmer Archipelago (Arenz *et al*., 2010), Terra Nova Bay and Edmonson Point (Gesheva, 2009), MacRobertson and Enderby Lands (Fletcher, 1985), Ongul Islands (Tubaki, 1961), McMurdo Sound (Tubaki and Asano, 1965), the Dry Valleys (Cameron, Morelli and Honour, 1973), Windmill Island (Azmi and Seppelt, 1997), Taylor Valley (Connell *et al*., 2006), the Antarctic Peninsula (Corte and Daglio, 1963), and Signy Island (Pugh and Allsop, 1982). Adams et al. (2006) also documented this species from Victoria Land from a bryophyte dominated site. However, mostly this species has been recovered from contaminated sites (Azmi and Seppelt, 1997) and moss-dominated sites (Bridge and Newsham, 2009).

Verticillium sp., which was the most common taxon obtained at 25°C in the present study, and is known as a tannase producer has been isolated from Signy Island (Heal, Bailey and Latter, 1967), MacRobertson and Enderby Lands (Fletcher, 1985), continental Antarctica (Fenice *et al*., 1998) and King George Island (Kasieczka-Burnecka *et al*., 2007). Fungi from this genus have been isolated mainly from soils, and also from other substrata such as plants, moss, insects and nematodes (Tosi *et al.,* 2002; Fahleson, Hu and Dixelius, 2004). *Verticillium* sp. was only found in human impacted sites in the current study, which may indicate that this species posseses a potentially interesting association with antropogenic activity, for instance as a food spoiling agent.

The present study recorded *Thelebolus* sp. from the pristine site. As noted above, this site was dominated by teleomorph ascomycetes. *Thelebolus microspores*, which is known to be a coprophilic fungus, has been isolated from MacRobertson Land, Ross Sea (Arenz *et al*., 2010), Bunger Hills, Sabrina Island and the Vestfold Hills (Arenz *et al*., 2010) and Victoria Land (Adams *et al*., 2006). Other species of *Thelebolus* have been isolated from MacRobertson and Enderby Lands (Fletcher, 1985). *Thelebolus psychrophilus* was previously isolated from soil, plants and mostly from dung in the Northern Hemisphere temperate zone (Brunati *et al*., 2009). Certain species of *Thelebolus* are believed to have been introduced to Antarctica by vectors like migratory birds (de Hoog *et al.,* 2005).

Mrakia frigida, which was isolated from the human impacted site in the current study was also previously reported from bryophyte dominated sites in Victoria Land (Adams *et al*., 2006), benthic mats of Antarctic lakes from the Larsemann Hills, Vestfold Hills and McMurdo Dry Valleys (Brunati *et al*., 2009), soil from King George Island (Xin and Zhou, 2007), and snow from the Larsemann Hills, Vestfold Hills and McMurdo Dry Valleys (Barnett, Payne and Yarrow, 1990). This species has also been documented from non Antarctic regions including Alpine glacier cryoconites and Siberia (Margesin *et al*.,

2005). However, this species was not common in the human impacted lake shore site in the present study.

Mortierella sp., which is psychrotolerant, recorded 11.3% of the fungal occurrences in the current study. It was isolated from all sampling sites except the pristine site. This species has been reported as a common soil fungus in the Antarctic region (Bridge and Worland, 2008). Previously it has been reported from the Victoria Land (Adams *et al*., 2006), Terra Nova Bay and Edmonson Point (Gesheva, 2009) and Palmer Archipelago (Arenz *et al*., 2010). Usually this species is isolated from damp and moss-covered soils in Peninsular and continental Antarctica (Adams *et al*., 2006; Bridge and Newsham, 2009). *Mortierella* sp. are also well known to degrade hydrocarbons, which may explain why this taxon was found abundantly near the human impacted site, which has a high possibility of oil contamination.

Phialophora was previously reported from McMurdo Sound region (Aislabie *et al*., 2001; Arenz *et al*., 2006), Victoria Land (Adams *et al*., 2006), Terra Nova Bay and Edmosnson Point (Gesheva, 2009) and Windmill Island (Gesheva, 2010). This species' occurrence is related to oil contamination, and it has been found to effectively degrade the hydrocarbon pyrene (Ravelet *et al*., 2000). We found four different strains of this genus from the human impacted lake shore site and one from the pristine site.

Fusarium sp. has been isolated from Schirmacher Oasis (Singh *et al*., 2006), a human impacted site at Windmill Island (Azmi and Seppelt, 1997; Bradner *et al*., 2003) and the Ross Sea region (Arenz *et al*., 2006; Duncan *et al*., 2008). In previous studies this taxon has always been found to be less frequent, for instance 3% in Arenz *et al*. (2006). In the present study, *Fusarium* sp. was isolated from the station site. This is consistent with Azmi and Seppelt (1997). Members of this genus are also known to include agricultural plant and animal pathogens (Hoshino, Xiao and Tkachenko, 2009).

Species richness is an important element of biodiversity in the soil (Arenz *et al*., 2006). Our study revealed that species richness, as indicated by the Shannon Diversity Index values, at the human impacted lake shore, ornithogenic, pristine and station sites were 5.63, 1.85, 3.40 and 3.09 respectively. Fletcher (1985) suggested that fungal occurrence is associated with the presence of organic material. Therefore, the presence of certain taxa at a specific site should be related to the nature of the site. When the nutrient or substrate levels are higher, then the chances for fungi proliferation and increased diversity are also higher.

The abundance of fungi is high in sites which are influenced by vegetation. This probably results from C and N supply to the soil from the vegetation, itself encouraged by higher moisture levels in the soil (Arenz and Blanchette, 2011). An important function of fungi in this ecosystem is to degrade plant material, by breaking down polymers such as cellulose from plants.

The wide diversity of fungi occurring in soils perform different ecological functions (Meyling, 2007). Different groups of fungi require different techniques and media for their isolation. Direct soil plating methods recommended by Bills *et al*. (2004) for soil fungi isolation include the suspension plating method (dilution method), particle filtration method and Warcup soil plating method. The current study used only one technique, the Warcup soil plating method. It is clearly possible that a higher number of fungi would have been obtained if additional methods were used (Bills *et al.,* 2004). Potato Dextrose Agar (PDA) was used to isolate and culture in this study, and was chosen as it is known to be a good media for fungal growth studies (Azmi and Seppelt, 1997) and is widely used (e.g. Hughes *et al*., 2003; Yergeau *et al.*, 2007; Brunati *et al*., 2009).

The present study reported a greater proportion of psychrophilic species than psychrotolerant. This is in contradiction with previous Antarctic reports which have suggested that psychrotolerant species are more frequent (Vishniac, 1993; Onofri *et al.*, 2004; Ruisi *et*

al., 2007; Ludley and Robinson, 2008), although the current study is based on a relatively small number of species overall.

4.2 Assessment of extracellular hydrolase enzymes

Vazquez *et al*. (2008) reported microbial enzyme production from bacteria from Jubany Station, King George Island previously. However, to date there is no report of enzymes from fungi from this Island. In the present study, filamentous fungi (eg. *G. pannorum*) and yeasts (*M. frigida*) demonstrated good significant enzyme activity across all the enzymes screened. These enzyme producers are suitable targets for further investgation of their enzyme properties and potential for exploitation.

4.2.1 Amylolytic activity of soil microfungi

This study is the first report of amylase from soil microfungi from King George Island. Of the strains isolated, 57.1% of taxa showed activity towards amylase. The majority of the strains (10) showing activity for amylase were attributable to *G. pannorum*. Beside this species, two anamorphic ascomycetes (*Penicillium* sp. and Ascomycete sp.17), a basidiomycete (*Aureobasidium* sp.), a mitosporic fungus (Mitosporic fungi sp.18) and yeast (Yeast sp. 16) showed activity for amylase. Of these strains, eight were obtained from the human impacted lake shore, one from the ornithogenic site, two from the pristine site and five from the station site. Overall, 12 of the strains were psychrotolerant and four psychrophilic. In terms of relative activity levels, significant activity of amylase was demonstrated by five psychrotolerant and two psychrophilic taxa.

A study from Terra Nova Bay, Victoria Land, continental Antarctica reported 54.5% of strains to show amylase activity from the fungi isolated, with *G. pannorum* recording the highest activity (Fenice *et al*., 1997). They reported five different strains of *G. pannorum v. pan*. and all showed positive results in amylase screening. However, isolation temperature in their screening was 25°C, which differs from the 4°C of the present study.

The three strong enzyme producing strains chosen for the amylase assay in the current study all represented *G. pannorum*. They were each obtained from different sampling sites, with that isolated from the ornithogenic site having the highest specific activity (3.208U/mg). The ornithogenic site is likely to include abundant glycogen derived from the local bird population (Highley, 1997). The other two strains of *G. pannorum* were obtained from human influenced sites, one from the human impacted lake shore and the other from the station site. The highest rate of glucose production (0.06 µmol/ml/min) was achieved by the strain isolated from the ornithogenic site. The other two strains yielded slightly lower rates of glucose production in comparison. *G. pannorum* clearly has potential as a producer of amylase and its development for exploitation should be explored further.

Fenice *et al*. (1997) documented no amylase activity for *Aureobasidium* sp., while we found a strain of this genus to posses significant activity. Conversely, Fenice *et al*. (1997) found *Thelebolus microsporus* to possess positive activity while the present study did not. However, as well as the differences between the studies in isolation and incubation conditions, there is clearly also a large possibility that the strains obtained belong to different species.

4.2.2 Cellulolytic acvity of soil microfungi

The present study is the first report of cellulase activity in soil microfungi from King George Island. Twelve strains showing this activity were obtained from the human impacted lake shore, one from the ornithogenic site, five from the pristine site and five from the station site. Twelve psychrotolerant and 11 psychrophilic strains showed cellulolytic activity, with three and four strains, respectively, exhibiting significant levels of activity. The highest RA value was recorded for two *G. pannorum* strains and *M. frigida* which were isolated from the pristine site, station site and human impacted lake shore, respectively. This is consistent with a study from Discovery Hut, Hut Point, Ross Island, which reported *G. pannorum* to able to degrade cellulose (Duncan *et al*., 2008). Strains of *M. frigida* are often isolated from moss covered areas (Adams *et al*., 2006), but in the current study the species was also isolated from the human impacted lake shore.

Soils from human impacted areas tend to have higher cellulose contents through contribution from waste materials (Joseph *et al*., 2007). There are many possible waste materials that can be introduced into the environment in Antarctica, in particular including hydrocarbons from vehicle and other machinery fuels, and organic wastes from foods and packing materials. In a previous study from Victoria Land (Fenice *et al.*, 1997) it was reported that 36.4% of the strains obtained demonstrated cellulase activity. However, among five strains of *G. pannorum var. pan*., only one showed weak activity for cellulase (Fenice *et al*., 1997). This is in contrast with the present study, where all *G. pannorum* strains exhibited activity for cellulase and only one showed weak activity. Fenice *et al*. (1997) also screened *Aureobasidium* sp. and *T. microsporus*, but no activity was detected. In the current study cellulase activity was detected in *Aureobasidium* sp. but not in *Thelebolus* sp. As

above, this could be as a result of differences in the isolation and incubation temperatures between the two studies, or in strain identity. It is not possibly to compare the strength of the enzyme activity between the studies as Fenice *et al*. (1997) did not measure RA.

Cellulase activity was also reported by Margesin *et al*. (2003) from yeasts isolated from alpine glacier cryoconites, and various alpine, Siberian and Antarctic environments. However it is not possible to link specific isolates and locations from the information included in this publication. From 28 isolates of yeasts, only seven demonstrated activity for cellulase in Margesin *et al.* (2003). In the present investigation, two out of three isolates of yeasts exhibited cellulase activity.

Relative activity and quantitative measurement of cellulase activity was reported by Duncan *et al*. (2006) from the Cape Evans historic hut, Ross Island. There were three strains of *Geomyces* sp. reported and the RAs of these strains were 1.0, 1.33 and 1.42. These are somewhat higher than found in the present study, where majority of the strains exhibited RA below 1.0, with three exceptions - two strains isolated from the pristine site (1.1 and 1.2) and one from the station site (1.2).

Duncan *et al.*'s (2008) screening test reported that 61% of fungal isolates from Discovery Hut, Hut Point, Ross Island showed cellulase activity, while in the current study 82.1% of the strains obtained showed activity. The only genus common to both studies was *Geomyces.* All four *Geomyces* strains obtained by Duncan *et al*. (2008) demonstrated cellulase activity, as was the case here. In this case, both studies used an incubation temperature at 4°C. The strength of enzyme activity reported by Duncan *et al*. (2008) was very similar with those of the current study, ranging from 0.5-1.68 in comparison with the present study's 0.2-1.2.

Quantification of cellulase activity from Antarctic fungi has previously been reported by Hurst *et al*. (1983) and Duncan *et al*. (2006, 2008). Fenice *et al*. (1997) reported

screening results but no quantification of activity levels was carried out. *Geomyces pannorum* isolated from the ornithogenic site in the current study showed activity in the range 0.198-0.251U/mg. Duncan *et al*. (2006) reported, there is no exact number stated regarding specific activity but estimated 8-59 U/mg from the graph they provided. Duncan *et al*. (2008) quantified cellulase production, finding that *Geomyces* sp. possessed a remarkable amount, ranging from 17.1 to 63.7 U/mg, in a study using both 4°C and 15°C culture temperatures for quantification. In their study some strains showed higher activity at 4°C and others at 15°C although (and as with the current study which only used 4°C), no attempt to assess the true optimum temperature for enzyme activity was made.

4.2.3 Protease activity of soil microfungi

This study's identification of protease activity is also the first report from soil microfungi from King George Island. Protease activity has been reported from soil bacteria isolated near Jubany Station on King George Island (Vazquez *et al*., 2008). In the current study 13 psychrotolerant and eight psychrophilic fungal taxa possessed protease activity, of which nine psychrotolerant and three psychrophilic taxa showed significant protease acitivity. Nine were isolated from the human impacted lake shore, one from the ornithogenic site, five from the pristine site and five at the station site. Fenice *et al*. (1997) reported 21.2% isolates showing protease activity, in contrast with the current study's 75% of isolates showing activity.

The three isolates showing highest protease activity were all attributed to *G. pannorum*. This contrasts with Fenice *et al*. (1997), who reported four strains of *G. pannorum var. pan.* to posses no protease activity for protease (although again note that the two stud-

ies are not directly comparable, as they involved different incubation temperatures and possibly different strains). Fenice *et al*. (1997) also reported a negative result for protease activity in *Aureobasidium* sp. and *T. microporus*, while the current study documented positive results for both genera.

Thelebolus sp. which is a psychrophilic strain, did not show any appreciable production of any of the three enzyme types examined. Members of this genus are also known as being least productive in antimicrobial activity in comparison with cosmopolitan fungi such as *Aspergillus, Penicillium* and *Cladosporium* (Brunati *et al*., 2009).

Margesin *et al*. (2003) reported six of 28 yeast isolates exhibiting protease activity, while the present study identified activity in two of the three yeast isolates obtained. However, no genera were common to both studies. A *Penicillium* strain isolated in the present study demonstrated weak protease activity, consistent with Bradner *et al*.'s (1999a) suggestion that this genus is not a good producer of protease.

Extracellular protease activity was previously reported from Schirmacher Oasis, continental Antarctica (Ray *et al.,* 1992). That study did not screen protease activity, instead purifying the enzyme from *Candida humicola*. This is a psychrotolerant yeast and is active at temperatures ranging from 0 to 45°C. However this species was not isolated in the current investigation.

4.3 Concluding overview of the soil microfungi and enzyme production

Considerable numbers of soil microfungi have been isolated from King George Island in the present study, comprising representatives of psychrophilic, psychrotolerant and mesophilic thermal classes. While these habitats clearly experience chronically low tem-

peratures, various other aspects of temperature stress, including large and short term variation, also present stress challenges to the soil microbiota (see Peck *et al*., 2006 for discussion). Therefore, while our study focused on the psychrophilic and psychrotolerant fungal strains obtained, it is unsurprising that mesophilic fungi were also noted to be present (Krishnan *et al*., 2011).

Most of the strains identified in the present study are closely related to others already reported as being psychrotolerant or psychrophilic from various parts of Antarctica, including taxa such as *Mortierella*, *Antarctomyces*, *Thelebolus*, *Penicillium*, *M. frigida* and *G. pannorum* (Wicklow and Malloch, 1971; Zucconi *et al*., 1996; Robinson, 2001; Tosi *et al*., 2002; de Hoog *et al*., 2005; Vishniac, 2006; Brunati *et al*., 2009; Gesheva, 2009, 2010). The most frequently isolated taxa, *G. pannorum*, *Mortierella* sp. and *Verticillium* sp. clearly posses important ecological functions such as involvement in degradation of bird feathers or hydrocarbons, or as pathogens of plants, insects and nematodes (Marshall, 1998; April, Foght and Currah, 2000; Fahleson *et al*., 2004).

Overall, we found a greater number of psychrotolerant strains compared to psychrophilic, virtually all showing significant activity for extracellular hydrolase enzymes, which reinforces the importance of these enzymes in the extreme environments of Antarctica. Enzyme activity varied between strains, even though they could often be attributed to the same species. For instance, two different strains of *G. pannorum* from Antarctica, *G. pannorum* var *pannorum* and *G. pannorum* var. *vinaceus*, exhibited totally different metabolism (Finotti *et al*., 1996). These authors monitored glucose, lipid and amino acid consumption, respiratory rate and growth rate and observed that both strains exhibited different metabolic behavior. Similar differences may underly the different enzyme activity levels found in the present study.

Using Duncan *et al*.'s (2008) definition of 'significant activity' requiring an Relative Activity (RA) > 1, only one strain obtained in the current study showed significant activity across all three enzymes examined, that being *G. pannorum* (obtained from the station site). In terms of RA values, our data show that cellulose decomposition by cellulase shows generally greater activity than those of the other two enzymes examined.

Enzyme quantification indicated that amylase production from soil microfungi from King George Island was stronger in comparison with cellulase. The glycogen from animal and bird influence in this area may contribute to this.

Fungi with wide enzymatic patterns posses high eco-nutritional versatility (Cooke and Whipps, 1980). An example from the current study is given by *G. pannorum*. Eco-nutritional versatility can be defined as the capability to thrive in an asexual state and survive environmental changes which are generally harmful (Cooke and Whipps, 1980). Since the terrestrial microhabitats of Antarctica are usually characterized by the presence of low competition (Convey, 1996), this high nutritional versatility is likely to be a successful strategy (Fenice *et al*., 1997).

The present study has screened and quantified enzyme activity of soil microfungi from King George Island. Future studies should proceed with characterization and purification of these enzymes, in order to start to test and develop their application in biotechnology.

5.0 Conclusions

1. Forty-one fungal strains comprising 24 Ascomycota, eight mitosporic fungi, one Zygomycota, one Basidiomycota, four yeasts, two unidentified species and a sterile mycelium were identified from isolated cultured from different soils on King George Island, South Shetland Islands.
2. Species richness calculated using the Margalef Diversity Index indicated that the human impacted lake shore possessed the highest species richness (5.63), followed by the pristine site, station site and ornithogenic site with index values of 3.40, 3.09 and 1.85 respectively.
3. The Shannon Wiener Diversity Index also indicated that diversity of fungi was highest at the human impacted lakeshore site, and that the pristine site possessed the most even distribution.
4. The 41 fungal strains were categorized into three thermal classes: 16 were confirmed as mesophiles, including 12 Ascomycota and four mitosporic fungi; four psychrotolerant strains included three Ascomycota and one Zygomycota; 21 psychrophilic strains included nine Ascomycota, four mitosporic fungi, four yeasts, one Basidiomycota, two unidentified species and one sterile mycelium.
5. The most common fungi isolated in culture at 4°C from King George Island were *Geomyces pannorum* and *Mortierella* sp.
6. In culture at 25°C, *Verticillium* sp. and *Geomyces pannorum* were identified to be the most common species.
7. Enzyme screening revealed that 57.1% of the fungal strains possessed positive activity for amylase. The strongest activity was shown by *G. pannorum* isolated from

the station site with a relative activity (RA) value of 2.1, followed by strains of the same species isolated from the human impacted lake (1.8) and ornithogenic site (1.8).

8. Cellulase screening demonstrated that 82.1% of the fungal strains showed positive activity with the strongest RA recorded by *M. frigida* from the human impacted lakeshore (1.7), and *G. pannorum* isolated from the station site (1.2) and pristine site (1.2).
9. Protease activity was shown by 75% of the strains examined. Significant RA was again recorded by *G. pannorum*, in two strains from the human impacted lakeshore (2.5 and 2.0) and one from the pristine site (2.2).
10. Amylase quantification for *G. pannorum* isolated from the ornithogenic, human impacted lakeshore and station sites showed specific activity of 3.208U/mg, 2.466U/mg and 1.209U/mg respectively.
11. *G. pannorum* from the pristine site showed the highest cellulase activity (0.213U/mg) followed by *M. frigida* (0.251U/mg) and *G. pannorum* from the station site (0.198U/mg).

Appendix A

Media preparation

Amylase screening media

R2A agar (Fluka Analytical)	7.25g
Starch	1.6g
Distilled water	400ml

Preparation

The above ingredients were mix together and sterilized at 120°C for 20 minutes.

Cellulase screening media

R2A agar (Fluka Analytical)	7.25g
Carboxymethylcellulose (CMC)	1.6g
Trypan Blue	0.04g
Distilled water	400ml

Preparation

The above ingredients were mix together and sterilized at 120°C for 20 minutes.

Protease screening media

R2A agar (Fluka Analytical)	7.25g
Skim milk	1.6g
Distilled water	400ml

Preparation

The above ingredients were mix together and sterilized at 120°C for 20 minutes.

Staining solution

Coomassie brilliant blue B	1.25g
Methanol	80ml
Acetic acid	20ml

Preparation

The above chemicals were mixed together and kept in a reagent bottle.

Enzyme assay

Amylase production media

Materials

KH_2PO_4	1.4g/l
NH_4NO_3	10g/l
KCl	0.5g/l

$MGSO_4.7H_2O$	0.1g/l
$FeSO_4.7H_2O$	0.01g/l
Soluble starch	20g/l

Preparation

The above ingredients were dissolved in 1000ml of distilled water and the pH was adjusted to 6.5. The mixed medium was then distributed to ten 250ml flasks and sterilized at 121°C for 20 minutes. The sterilized media were stored in 4°C refrigerator until further use.

Cellulase production media

Materials

Carboxymethylcellulose	10g/l
Peptone	1.0g/l
Urea	0.3g/l
$MnSO_4.H_2O$	0.016g/l
$ZnSO_4.7H_2O$	0.014gl
$(NH_4)2SO_4$	0.5g/l
$MgSO_4.7H_2O$	0.1g
$FeSO_4.7H_2O$	0.029g/l
$CaCl_2$	2.0g/l
$CoCl_2.6H_2O$	1.4/l
KH_2PO_4	0.3g/l

Preparation

The above ingredients were dissolved in 1000ml of distilled water and the pH was adjusted to 6.5. The mixed medium was then distributed to nine 250ml flasks and sterilized at 121°C for 20 minutes. The sterilized media were stored in 4°C refrigerator until further use.

Appendix B

Reagents

Bradford Reagent

Materials

Coomassie Brilliant Blue	10mg
Ethanol 95%	5ml
Orthophosphoric acid 85%	10ml

Preparation

The chemicals were mixed together and kept in amber bottle in 4°C. This reagent was filtered through Whatman No.1 filter paper prior to protein assay. This is stable for 1 month.

Dinitrosalicylic (DNS) Reagent

Materials

Distilled water	708ml
NaOH	9.9g
3,5- Dinitrosalycylic acid	5.3g
Sodium potassium tartarate (Rochelle salt)	153g
Phenol	3.8ml
Natrium metabisulphite	8.3g

Preparation

DNS acid and NaOH were dissolved in distilled water using a magnetic stirrer and bar and the other materials were slowly added to the solution. This solution is stable for 1 month.