Antarctic Microfungi as a Potential Bioresource

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Declaration

The research presented in this thesis is original work performed between March 1997 and March 2003 by the author. This research has not been submitted to any other university or institution as part of the requirements for any higher degree or course.

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Abstract

The Antarctic occupies that region of the planet that falls below the 60th parallel of South latitude. Although it has been frequented by adventurers, journeyman scientists and tourists for the past 100 years, the Continent has remained virtually unoccupied. The intense cold, the absence of human occupation and the limited range of local higher animal species have combined to create the impression that the Continent is virtually devoid of life.

Although the microbiota of the Antarctic has attracted some small level of attention in the past, the examination of filamentous microfungi has been largely overlooked and fallen to a small group of dedicated investigators. In this study it will be shown that far from being an insignificant component of the Antarctic network, microfungi represent a potentially large and so far untapped bioresource.

From just 11 bryophyte samples collected at four sites in the Ross Sea/Dry Valleys region of Southern Antarctica, some 30 microfungal isolates were recovered. Using molecular techniques, the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA (nrDNA) was sequenced to reveal no less than nine unique microfungal species. For only two of these species did the ITS sequence data produce a 100% match with records held on the public databases. This investigation also highlighted the problems inherent in the traditional morphological identification system which are now being perpetuated in the molecular database records.

A set of seven notionally identified isolates obtained from ornithogenic soil samples gathered in the Windmill Islands in Eastern Antarctica (offshore from the Australian

Antarctic Division's Casey Station) were also subjected to molecular identification based on ITS sequence data. Each of the seven isolates was identified as a unique species; six were cosmopolitan in nature and the one remaining bore very little resemblance at the molecular level to any of the recorded species although it was provided with an epithet commonly used in the identification of Antarctic microfungal species.

To evaluate their potential as a bioresource, samples of Antarctic microfungi were examined to determine if the same physiological factors common to mesophilic species also applied to their Antarctic analogues. It is known that when placed under stress, trehalose can act as a protectant against cold (cryoprotection) and dehydration in mesophilic yeasts and fungi. The level of trehalose produced by the Antarctic isolates and their mesophilic analogues when subjected to stress was compared. A similar comparison was made for the production of glycerol which is well established as a compatible solute providing protection to mesophilic species against osmotic stress. Only in the case of trehalose production by an Antarctic *Embellisia* was there any indication that either of these two compounds could play a significant role in providing protection to the Antarctic fungi against the rigours of their environment, which leaves open to question what in fact does.

In the course of investigating the means by which Antarctic microfungi guard against the damage which can ensue when subjected to oxidative stress, flow cytometry was introduced as an investigatory tool. It was established that there is a window of opportunity during which flow cytometry can be used to undertake a detailed analysis of the early stages of fungal growth from germination through hyphal development.

Of major significance in determining the potential of Antarctic microfungi as a resource is their ability to produce new and novel enzymes and proteins. The microfungal isolates were screened for hydrolytic activity on solid media containing indicative substrates and proved to be a fruitful source of enzymes active over a range of temperatures. A detailed characterisation of two hemicellulases, β-mannanase and xylanase, secreted into a liquid medium by a subset of the Antarctic fungi and a high producing mesophilic reference strain permitted direct comparisons to be made. It was shown that the maximum hemicellulase activity of the Antarctic strains occurred at least 10ºC and as much as 30ºC lower than that of the reference strain and that mannanase activity for two of the Antarctic isolates exceeded 40% of their maximum at 0ºC. These assay results highlight the potential of Antarctic microfungi to yield novel cold-active enzymes.

As a final measure of the capacity of the Antarctic to yield novel enzymes from its microfungal stock, a lipase gene was selected as a target for isolation and expression in a heterologous fungal host. Using PCR techniques, the gene of interest was isolated from an Antarctic isolate of *Penicillium allii,* transformed into the mesophilic production host *Trichoderma reesei* and the active protein successfully produced in the growth medium. The recombinant lipase was assayed and found to exhibit novel characteristics consistent with a cold-adapted enzyme.

Preface

There are many people that should be acknowledged for the support they have given me over a long period of time. Firstly, I give my heartfelt thanks to my supervisor, Associate Professor Helena Nevalainen, for her friendship, guidance, encouragement over many years during the course of this study and throughout my undergraduate years. Her help and constructive criticism, particularly in the preparation of this document and other papers, was warmly appreciated. Special thanks also to my Associate Supervisor, Associate Professor Michael Gillings for his valued guidance in matters molecular, sequencing and in the preparation of this document and other papers.

I also extend my warmest thanks to all of my colleagues in the EDGE Laboratory at Macquarie University for providing such a pleasant working environment and in particular to Professor Peter Bergquist, Drs. Morland Gibbs, Junior Te'o, Roberto Anatori, Anwar Sunna and Noosha Ehya and Ms Roz Reeves and Ms Natalie Curach each of whom has helped me in some way during the course of this study, with either advice or constructive comment. I would also like to thank Professor Duncan Veal and Dr. Paul Attfield for their guidance and help with the flow cytometer, Dr Robert Willows for advice on biochemistry and my co-authors Dr. Philip Bell and in particular Dr. Rani Sidhu who provided invaluable assistance in the tedious task of assaying for hemicellulase activity.

Finally, I express my warmest thanks to my wife Dawn for her love, patience and support throughout this work and to my son Alexander who has helped me greatly with some of the more mystifying points of computer software.

List of original publications

This work is based on the following articles, referred to in the text by the Roman numerals given below. Additional unpublished data is also presented.

I Bradner, J. R., Gillings, M and Nevalainen, K. M. H. (1999). Qualitative assessment of hydrolytic activities in antarctic microfungi grown at different temperatures on solid media. *World Journal of Microbiology & Biotechnology*. **15**:131-132.

II Bradner, J. R., Sidhu, R. K., Gillings, M. and Nevalainen K. M. H. (1999). Hemicellulase activity of antarctic microfungi. *Journal of Applied Microbiology*. **87**:366-370.

III Bradner, J. R. , Sidhu, R. K., Yee, B., Skotnicki, M. L., Selkirk, P. M. and Nevalainen, K. M. H. (2000). A new microfungal isolate, *Embellisia* sp., associated with the Antarctic moss *Bryum argenteum*. *Polar Biology*. **23**:730-732.

IV Bradner, J. R. and Nevalainen K.M.H. (2003). Metabolic activity in filamentous fungi can be analysed by flow cytometry. *Journal of Microbiological Methods*. **54**:193-201.

V Bradner, J. R., Bell, P. J. L., Te'o, V. S. J. and Nevalainen, K. M. H. (2003). The application of PCR for the isolation of a lipase from the genomic DNA of an Antarctic microfungus. *Current Genetics* **44**:224-230.

The author of this thesis had the main responsibility for the work contained in each of these publications and also for planning the experiments and writing the articles. The role of Assoc. Prof. Helena Nevalainen (publications I – V) and Assoc. Prof. Michael Gillings (I & II) was to act in their capacity as my supervisors, providing me with overall support and guidance and participated in the planning and evaluation of the experiments. Dr Rani Sidhu provided technical assistance in assaying for hemicellulase activity (II) and together with Miss Beta Yee, assisted in the isolation of the fungal material associated with the bryophytes collected in Antarctica by Dr. Patricia Selkirk and Dr. Mary Skotnicki (III). Dr. Philip Bell designed the suite of PCR primers used to identify the lipase gene in the Antarctic microfungus and Dr. Junior Te'o was responsible for engineering the plasmid utilised in the transformation of the heterologous fungal host and provided support with the biolistic transformation system (V). Sequencing with the ABI Prism automated fluorescent DNA sequencer was undertaken by the the staff of the facility at Macquarie University.

Contents

Abbreviations

aa	amino acid
Ala	alanine
App	Appendix
Arg	arginine
Asn	asparagine
BAC	bacterial artificial chromosome
bp	base pairs
DHE	dihydroethidium (a fluorescent stain)
FC	flow cytometry/cytometer
FL	fluorescence
FSC	forward scatter
gDNA	genomic DNA
Glu	glutamic acid
HI	hexidium iodide
IPTG	isopropyl-β-D-thiogalactosidase
ITS	internal transcribed spacer region
Kbp	kilo base pairs
k_{cat}	dissociation rate (s^{-1}) - *Michaelis-Menten kinetics*
kDa	kilo Dalton
K_M	Michaelis constant (mol L^{-1}) - *Michaelis-Menten kinetics*
lat	latitude
LB	Luria-Bertani (medium)
L-broth	Luria-Bertani broth
long	longitude
Lys	lysine
nrDNA	ribosomal DNA (nuclear)
nt	nucleotide
PCR	polymerase chain reaction
PD	potato dextrose
PDA	potato dextrose agar
PMT	photomultiplier tube
RAPD	randomly amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
ROS	reactive oxygen species
R/T	room temperature
S.E.	Standard Error
sq km	square kilometre
SSC	side scatter
SSU	small sub-unit
uv	ultra violet light
UP-PCR	universally primed PCR
Val	valine
v/v	volume/volume
w/v	weight/volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactosidase

1. Introduction

The Antarctic continent lies mostly south of the Antarctic Circle and is the coldest, driest, windiest, highest, and most isolated of all the continents. Antarctica is a continent of ice which almost completely covers the continent connecting mountains, tablelands, archipelagos and seas. The total Antarctic surface area is estimated at 14 million sq km which in winter, when the sea ice is included, can expand to ca. 40 million sq km (Walton, 1984). Of this vast area, only 280,000 sq km is ice-free.

With the Antarctic Peninsula some 950 km away from South America, Antarctica is geographically isolated. Not only is it isolated geographically but it is also isolated climatically, being surrounded by the Antarctic Convergence – the border between cold Antarctic Ocean water (about -2ºC) and temperate oceans.

The governance and legal framework of Antarctica is essentially covered by the Antarctic Treaty with administration handled through meetings of the consultative member nations. At the end of 2001, there were 45 treaty member nations; 27 consultative (voting) and 18 non-consultative. Seven of the consultative nations have claims to portions of Antarctica as national territory (Fig 1.1). The Protocol on Environmental Protection which came into force in 1998 provides for the protection of the Antarctic environment whilst allowing for scientific research including the microbiota discussed in this work.

1.1 The Antarctic environment

Antarctica is about 98% thick continental ice sheet and 2% barren rock with average elevations between 2,000 and 4,000 m above sea level. Mountain ranges reach up to nearly 5000 m and ice-free coastal areas include parts of southern Victoria Land, Wilkes

Land, the Antarctic Peninsula area and parts of Ross Island on McMurdo Sound (Fig 41.1). Glaciers form ice shelves along about half of the coastline. The McMurdo Dry Valleys, at lat 76º30' – 78º30' S, long 160 – 164º E, form the largest (approximately 4800 sq km) of the ice-free areas on the continent and are one of the most extreme terrestrial environments on Earth (Fountain *et al.*, 1999; Priscu, 1998).

The mean temperature in the maritime Antarctic (West Coast of the Antarctic Peninsula and surrounding islands) during the warmest period is above freezing whereas in other coastal regions and in the McMurdo Dry Valleys the average remains slightly below freezing. However, during December and January the surface soil (0-20 cm depth) temperatures in the McMurdo Dry Valleys are consistently above freezing (Vincent, 1989). Most of the biological and fungal sampling in the Antarctic has occurred in the Peninsula, along other coastal regions and in the McMurdo Dry Valleys.

The katabatic (gravity-driven) winds which blow coastward from the high interior, allied with rainfall varying from 100 - 150 mm along the coast and water available as melted ice for only a few days in the year, make the Antarctic the driest and windiest of continents and place the microorganisms of the region under extreme environmental stress. Various climates prevail in Antarctica and microclimates are even more differentiated. It is characteristic of the continent that microclimates and nanoclimates are very variable and unstable with regards to temperature and water availability, a crucial requirement for microbial life (Walton, 1984).

The conditions of temperature and dryness, at least in the ice-free McMurdo Dry Valleys

Figure 1.1 The Antarctic Region (From 2000 World Factbook: Antarctica - U.S. CIA)

or Ross Desert are comparable to those prevailing on Mars. Soils in the Ross Desert are the most extreme cold habitat on Earth, multiply stressing the indigenous microorganisms with low temperature, aridity, high mineral-salt content, low substrate-carbon content, and very dubious photosynthetic productivity (Vishniac, 1993).

Due to the high latitude of the continent, considerable differences in the levels of solar radiation and day length occur between summer and winter. In mid-summer, within the Antarctic Circle, 24 h of daylight prevails and conversely in mid-winter there is total darkness. Antarctica is the most uv-irradiated region on Earth potentially adding further to the already high levels of oxidative stress faced by the local microbiota.

1.2 Antarctic inhabitants

Life is tenacious, it seems able to gain a foothold whatever the circumstances and Antarctica is no exception. The biology of the Antarctic, more so than on any other continent, is dominated by microorganisms (microspecies). Higher life forms - plants and animals (macrospecies), have also managed to gain a presence on the continent.

1.2.1 Macrospecies

The higher life forms that are found on the Antarctic continent can be grouped together into one of two categories. Firstly there are those species which are indigenous to the continent and secondly, those that are aliens.

1.2.1.1 Indigenous species

Within this category can be included all of the creatures which appear to spend the whole of their life cycle within the confines of the Antarctic continent and its surrounding seas.

A number of higher animals can be deemed to fall within this rather loose definition. The various penguin species which maintain their rookeries on the continental mainland, immediate offshore islands and continental ice sheets and also a number of seal and walrus species which likewise breed within the confines of the continent can be included. Most of these animals appear to spend some of the time, outside their breeding periods (usually winter), off the Antarctic continent possibly even moving into temperate waters. Notwithstanding that these animals may travel considerable distances beyond the Antarctic coast, their home remains Antarctica.

A few other bird species in addition to penguins appear to be permanent residents of the Antarctic continent. These include the Brown skua, Antarctic and Snow petrel, the Kelp gull and some species of tern. These birds nest on the ice free regions of the continent or adjacent islands, and appear to winter at sea on the surrounding pack-ice.

The only vegetative material to be identified on the Antarctic continent below the Peninsula are the cryptogams (mosses, liverworts and lichens). Two species of vascular plants, *Colobanthus quitensis* and *Deschampsia antarctica*, have been identified in the ice-free region of the Antarctic Peninsula down to approximately lat 68ºS (Alberdi *et al.*, 2002) but do not extend into continental Antarctica (Greene & Holtom, 1971).

1.2.1.2 Alien species

Many of the bird species found in the Antarctic environment fail to meet the above definition of an indigene invariably migrating during the winter months to the sub-Antarctic islands and beyond.

Probably the most obvious alien species to enter the Antarctic environment is man. Human exploration of the Antarctic continent began in the early years of the 19th century and continues to this day albeit now in the form of scientific research stations. Allied to the expansion of man into the region was the introduction of his work animals (now banned under the Treaty), dogs and occasionally larger animals such as ponies.

An inevitable consequence of this entry onto the continent by non-indigenous species is their ability to act as a vector for the transmission of alien microorganisms to the Antarctic.

1.2.2 Microspecies

There is abundant evidence for the presence of microorganisms on the Antarctic continent (Nichols *et al.*, 1999; Vincent, 1989; Vishniac, 1993); the biology of Antarctica, more than any other continent is dominated by microorganisms. Within the Antarctic framework, microspecies fall into a number of categories – invertebrates, bacteria (including archaebacteria), algae, cryptogams, yeasts, and filamentous fungi. The origins of the Antarctic microbiota is essentially unknown, without doubt much is indigenous. There are, however, a number of potential avenues by which alien species can be introduced to the region. As indicated above (1.2.1.2), the presence of migratory macrospecies provides a ready vehicle to carry foreign material to the continent. Man, his animals and his materiel, who throughout the ages and across all regions have been the harbingers of disease and destruction, provide an ever present source of ‘infection’ to the present day. Possibly the greatest source of contamination of the Antarctic wilderness is the wind. Various studies have been undertaken to assess the presence of airborne propagules in antarctic air (Corte & Daglio, 1994; Marshall & Chalmers, 1997; Sieburth, 1965), and a recent detailed study over a 13.5 month period by Marshall (1997b), albeit

on Signy Island in the sub-Antarctic South Orkney group, clearly demonstrated the ability of the wind to transport fungal spores.

1.2.2.1 Invertebrates

The Antarctic continent and environs support a range of invertebrates including mites, springtails, nematodes, rotifers and tardigrades (Courtright *et al.*, 2001; Porazinska *et al.*, 2002; Treonis *et al.*, 2002). These invertebrates survive among growths of cryptogams (cyanobacteria, green algae, lichens and bryophytes), in accumulations of detritus around penguin rookeries and seal wallows, in beach wrack and in the thin mineral soils (Block, 1984; 1992). Antarctic springtails and most mites are thought to feed primarily on fungi, lichens, algae, detritus and decaying plant matter, however, some mites are predatory and feed on any animal they can overpower. Four nematode species have been identified from soil samples collected in and near penguin rookeries on Ross Island (Cape Bird, Cape Crozier and Cape Royds) (Porazinska *et al.*, 2002), and a three species collected from within the McMurdo Dry Valleys (Courtright *et al.*, 2001).

1.2.2.2 Microflora

There are about 130 species bryophytes species in the Antarctic (Alberdi *et al.*, 2002) divided amongst the mosses and the hepatics, or liverworts. The bryophytes, which have been identified as a rich source of fungal material (Azmi & Seppelt, 1998; Greenfield & Wilson, 1981; Möller & Dreyfuss, 1996; Skotnicki *et al.*, 1996; Tosi *et al.*, 2002) can be found in almost all areas capable of supporting plant life in the Antarctic (Fig 1.2). Although not as widespread as the lichens, mosses have been collected from as far south as lat 84°30' in Southern Victoria Land. Moss lawns often occur on melt streams from glaciers, such as the Taylor Glacier in the Dry Valleys, Southern Victoria Land.

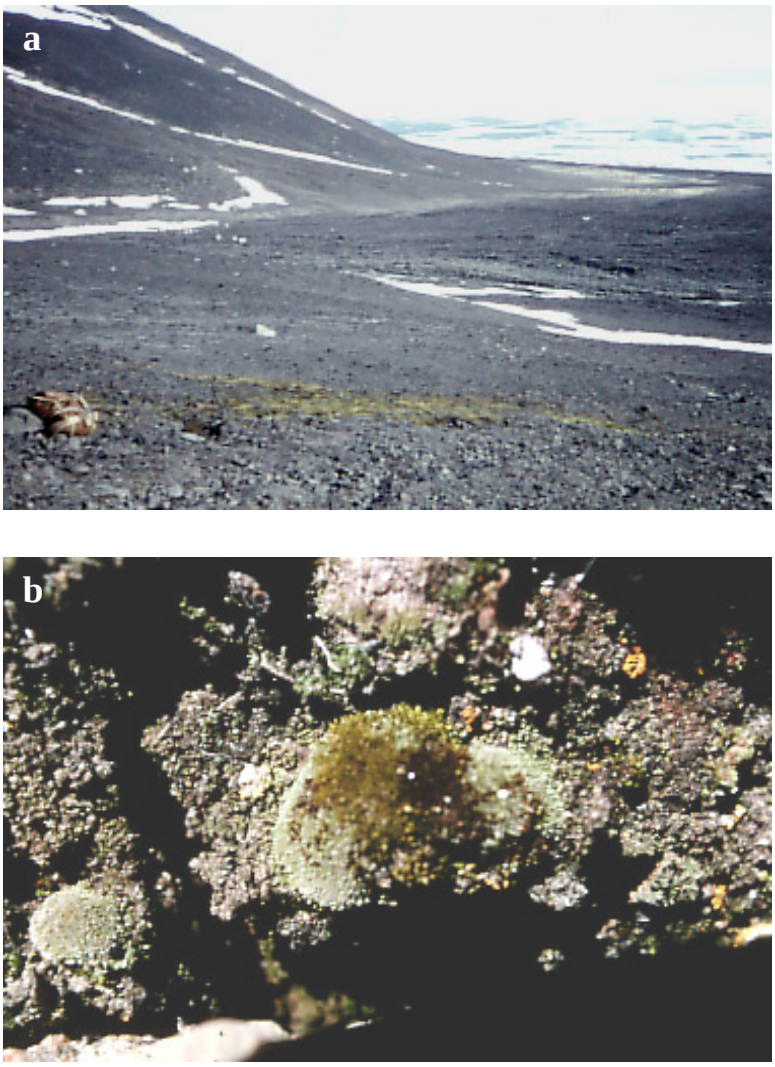

Figure 1.2 Antarctic bryophytes

The species of bryophytes illustrated here were the source of microfungal material isolated in this study (see Table 2.1)

a A lawn of *Hennediella heimii* at Cape Bird on Ross Island in the Ross Sea.

b *Bryum argenteum* and *Hennediella heimii* at Granite Harbour, Dry Valleys, Southern Victoria Land.

Photographs by Dr. P. M. Selkirk. Reproduced with permission.

1.2.2.3 Lichens

There are around 380 species of lichen in the Antarctic (Alberdi *et al.*, 2002) and they are one of the most conspicuous elements of the terrestrial ecosystem. Lichens, the symbiotic association of certain fungi and (usually) green algae, exist as three main types in Antarctica: crustose lichens which form a thin crust on the surface of the substrate they grow on, foliose lichens which form leaf like lobes and fruticose lichens which have a

shrubby growth habit. Lichens co-exist with bryophytes and vascular plants as equal partners in the most favourable areas of the Maritime Antarctic (Kappen, 2000). In areas with more extreme environmental conditions, lichens, after microorganisms and unicellular algae, are pioneers in occupying terrestrial habitats.

1.2.2.4 Filamentous fungi and yeasts

The distribution of fungi in Antarctica is most often associated with strong biotic influences such as birds (penguin rookeries) and vegetation (bryophyte and lichen communities). Within the Antarctic environment, moss is one of the micro-habitats richest in microfungi (Tosi *et al.*, 2002). At the time of Vishniac's publication (1993) he was able to publish a list of 82 taxa representing macro- and microfungi recorded in the Antarctic and most of the filamentous fungi and yeasts listed were cosmopolitan species. More recently (1998), about 230 different species of fungi have been recorded in maritime and continental Antarctica excluding sub-Antarctic (Onofri, 1998), some 50 odd appear to be new Antarctic species, the remainder are cosmopolitan (Onofri *et al.*, 1999).

Mycological investigations within the Antarctic region have largely centred upon the Antarctic Peninsula and the McMurdo Dry Valleys region of southern Victoria Land (Vishniac, 1993). Latterly, Azmi and Seppelt (1998) have conducted an extensive investigation of microfungi in the Windmill Islands region of Wilkes Land (the region occupied by the Australian Antarctic Division's Casey Station), Tosi *et al.* (2002) in mid-Victoria land (Terra Nova Station) and Aislaby *et al*. (2001) in petroleum polluted areas in the Ross Sea (Scott Base and Marble Point) and Wright Valley (Dry Valleys) region. Outside of these areas, mycological studies have, over the years, been reported from a number of sites around the Antarctic continent: Cape Hallett (Wicklow, 1968), Bunger Hills (Barker, 1977), Mac Robertson and Enderby Land (Fletcher *et al.*, 1985; Kerry, 1990a),

Roberskollen, Dronning Maud Land (Ryan *et al.*, 1989), the Vestfold Hills (Kerry, 1990b) and northern Victoria Land (Montemartini Courte & Gestro, 1994; Onofri *et al.*, 1994).

1.2.2.5 Other microorganisms

The remaining significant groups of organisms that make up the microbiota of the Antarctic are algae and bacteria. Collectively, these two groups probably represent the bulk of the organic material present on the continent. Although an abundance of bacterial material has been isolated from the Antarctic, relatively few have been described – approximately 30 species of bacteria and three species of *Archaea* (Nichols *et al.*, 1999).

On the Antarctic continent and subantarctic islands, algae live in lakes and streams, on moist soil and in snow banks. On the Antarctic continent, some algae also live in the spaces between the grains of porous sandstone rocks and underneath translucent quartz rocks where moisture and light are available for their growth. Soil algae, together with their associated bacteria, are ecologically important as they contribute to the organic material and help bind the soil particles together with the mucilage and slime they secrete. In summer, the algae accumulate in sufficient numbers to colour the snow banks red, green, orange and even grey. There are currently some 700 recorded species of terrestrial and aquatic algae in Antarctica.

1.3 Microfungi

As previously indicated, the distribution of fungi in the Antarctic is related to the distribution of 'hosts'. Microfungi, so called because most of the fungal body consists of microscopic threads extending through the substrate in which it grows and little, if any, of the fungus is visible at any one time, have been isolated from bryophyte and lichen communities, algal mats, soils and even from the carcasses of mummified seals.

In the main, Antarctic microfungi have been found to be psychrotrophic (mesophilic psychrotolerent) rather than psychrophilic (Azmi & Seppelt, 1997; Kerry, 1990a; Zucconi *et al.*, 1996). Both psychrophilic and psychrotrophic fungi have the ability to grow at 0ºC. Psychrophilic fungi have an optimum temperature for growth around 15ºC or lower, and a maximum of 20ºC or below, whereas psychrotrophic fungi have a maximum temperature for growth above 20ºC (Robinson, 2001).

Of particular interest to this study are microfungi identified as being associated with bryophytes and also those isolated from ornithogenic soils.

1.3.1 Fungi associated with ornithogenic coastal soil

Limited investigations have been undertaken into the incidence of microfungi in ornithogenic soils. The principal investigators to date have been Azmi and Seppelt (1998) who undertook a broad-scale study of microfungi in the Windmill Islands region of coastal Wilkes Land (centred on lat 66º17´S, long 110º32´E). In the course of that study some 361 isolates from 23 taxa were isolated from mineral soils in the vicinity of penguin and other bird colonies.

1.3.2 Fungi associated with bryophytes

The association of microfungi with bryophytes, particularly the mosses, in continental Antarctica and on the Peninsula and offshore islands, has been the subject of a somewhat greater amount of investigation (Azmi & Seppelt, 1998; Greenfield & Wilson, 1981; Möller & Dreyfuss, 1996; Tosi *et al.*, 2002). In the Windmill Islands, Azmi and Seppelt (1998) identified 237 isolates from 21 taxa from three moss species, Tosi *et al*. (2002) obtained from eight mosses 120 isolates representative of 28 species belonging to 18 genera collected in the area around the Italian Terra Nova Station in East Victoria Land.

From the vicinity of the Polish research station 'Arctowski' and the Argentinean station ' Jubany' on King George Island in the South Shetland Group, Möller and Dreyfuss (1996) recorded 55 species from 10 mosses (Table 1.1).

Table 1.1 Fungal genera reported to be associated with Antarctic bryophytes

Acremonium [2,3]	*Alternaria* [1]	*Arthrobotrys* [3,4]
Arthroderma [2]	*Aspergillus* [1]	*Aureobasidium* [1,4]
Cadophora [4]	*Chaetomium* [4]	*Chalara* [2]
Chaunopycnis [2]	*Chrysosporium* [1,3]	*Cladosporium* [1,4]
Conidiobolus [4]	*Cylindrocarpon* [2]	*Epicoccum* [1]
Geomyces [2,3,4]	*Monascella* [2]	*Monocillium* [2]
Mortierella [1,2,3,4]	*Mucor* [1,3]	*Mycelia sterilia* [2,3]
Myrioconium [2]	*Nectria* [3]	*Paecilomyces* [2,4]
Penicillium [1,2,3,4]	*Phaeosphaeria* [2]	*Phaeoseptoria* [2]
Phialophora [1,2,3]	*Phoma* [2,3,4]	*Phomopsis* [2]
Pycnostysanus [2]	*Rhodotorula* [4]	*Scolecobasidium* [4]
Thelebolus [2,3,4]	*Tolypocladium* [2]	*Trichoderma* [1]
Verticillium [4]		

[1] Greenfield and Wilson (1981); [2] Möller and Dreyfuss (1996); [3] Azmi and Seppelt (1998); [4] Tosi *et al.* (2002)

1.4 Identification of microfungi

It has already been noted above that a wide range of microscopic life forms including bacteria, fungi, microalgae and mosses colonise Antarctica where they form an integral part the Antarctic ecosystem and play a significant role in the energy flow, transformation of organic matter and soil productivity (Vincent, 1989; Vishniac, 1993). Filamentous fungi are found in all climatic zones ranging from the poles to the tropics. Previous reports describe over 150 fungal species isolated from soil and water samples collected in continental Antarctica (reviewed in Onofri *et al*., 1994 and discussed in 1.3 above). The focus of these studies has mainly been on the distribution and identification of fungi isolated from different Antarctic environments varying in salinity, humidity and nutrient

content (Azmi & Seppelt, 1998; Kerry, 1990a; Sugiyama *et al.*, 1967; Toyoda *et al.*, 1985). To date, very little is known about the origins of Antarctic filamentous fungi. There is a general theory that many hyphomycetes are not native to the Antarctic but have been landed there through the agencies of birds, animals (including man) or have been carried onto the continent as propagules on the wind (see 1.2.2 above). Generally, Antarctic species have been supplied their taxonomic epithet based on their morphological attributes which in many instances are analogous to their cosmopolitan namesakes.

Various methods are available for the identification of microfungi. The traditional method of identifying a fungal isolate is via a detailed examination of its morphological characteristics. More recently, the techniques of molecular biology are being applied.

1.4.1 Identification based on morphology

This technique for the identification of fungal species is well established and by and large forms the basis of the current fungal taxonomy. The principles for an identification of an isolate involve a series of measurements of specific features and observations of characteristics which can be used as keys to compare an unidentified isolate with type or authentic strains of standard reference species. Examples of the sort of morphological characteristics that may be taken into consideration are: conidiophore length; conidium size, shape, colour and surface texture; colonial morphology on different growth medium; pigmentation of growth medium and exudates. A detailed list of keys for fungal identification can be seen in Domsch *et al*. (1980) or Pitt (1979).

Although the morphological method of identification has generally proved adequate, it faces the drawbacks that it is expensive, requires highly skilled experts and may prove

unreliable due to the absence of a consistent phenotypic concept of what constitutes a particular species (Esposito & da Silva, 1998). Morphological taxonomy can, in part, be subjective and results can be influenced by such things as vagaries in the constituents of commercial growth medium.

1.4.2 Identification based upon molecular techniques

At the beginning of the last decade of the 20th century, Kohn (1992) observed what she described as a 'watershed period' in fungal systematics, noting that relatively few mycologists were doing taxonomy. She further noted that increasing numbers of mycologists were undertaking systematic studies of fungi by analysing samples of isolates to define groupings within populations, delimiting species, accommodating species in genera and seeking phylogenetic relationships among taxa. This avenue of research has continued using various combinations of DNA based techniques as can be illustrated by just a few examples such as investigations into *Trichoderma* (Bulat *et al.*, 1998; Dodd *et al.*, 2000; Fujimora & Okuda, 1994; Gams & Meyer, 1998; Kindermann *et al.*, 1998; Kuhls *et al.*, 1997; Kullnig *et al.*, 2000; Lieckfeldt & Seifert, 2000; Lieckfeldt *et al.*, 1998; Meyer *et al.*, 1992; Ospina-Giraldo *et al.*, 1998; Zimand *et al.*, 1994), *Penicillium* (Boysen *et al.*, 1996; Boysen *et al.*, 2000; Skouboe *et al.*, 1999), ascomycetous truffles (O'Donnell *et al.*, 1997), *Epicoccum* (Arenal *et al.*, 2000) and *Oidiodendron* (Hambleton *et al.*, 1998).

There are a variety of molecular techniques available for fungal taxonomic enquiry. These fall into two broad categories – protein based and DNA based. Within the first classification, molecular characteristics can be detected based on proteins using isozyme electrophoresis, immunological techniques and direct amino acid sequencing (Maxson & Maxson, 1990; Micales *et al.*, 1986; Murphy *et al.*, 1990; Swofford & Olsen, 1990). In

the latter category, the range of molecular techniques available for mycological investigation has been enhanced by the development of the polymerase chain reaction (PCR) which allows specific and very sensitive detection and production of DNA fragments from nucleic acid material from a wide variety of sources (Arenal *et al.*, 2000; Bruns *et al.*, 1990; Foster *et al.*, 1993; Metzenberg, 1991; Viaud *et al.*, 2000). Some of the techniques which utilise PCR in fungal identification include randomly amplified polymorphic DNA amplification (RAPD) (Williams *et al.*, 1990), universally primed PCR (UP-PCR) (Bulat *et al.*, 1994), restriction fragment length polymorphism (RFLP) analysis and DNA sequence analysis (White *et al.*, 1990).

1.4.2.1 RAPD

With RAPD analysis, a PCR reaction is conducted using a short (9 – 10 nucleotides) single arbitrary primer. The primer finds homology in the template DNA in the PCR reaction and initiates extension. Keeping the annealing temperature low (35 – 37ºC) ensures that the primer sequence will randomly anneal to the template DNA. After 30 – 45 cycles the product can be run on an agarose gel, stained with ethidium bromide and viewed under uv light. Reproducible bands of different size can be visualised on the gel indicating real sequence differences in the DNA of different strains or species (Foster *et al.*, 1993).

1.4.2.2 UP-PCR

UP-PCR is a PCR fingerprinting method similar to RAPD. It allows DNA from an organism to be amplified without previous knowledge of the DNA sequences and multibanding profiles (fingerprints) to be generated with gel electrophoresis. UP-PCR makes use of higher annealing temperatures (50 – 55ºC) than RAPD, fast ramping and

longer primers (~17 mer) to produce enhanced reproducibility (Gillings & Holley, 1997; Bulat *et al.*, 1998).

1.4.2.3 RFLP

RFLP is a technique in which organisms may be differentiated by analysis of the patterns derived from the cleavage by restriction endonucleases of PCR amplified fragments of their DNA. If two organisms differ in the distance between sites of cleavage of a particular restriction endonuclease, often due to mutations adding or removing a site, the length of the fragments produced will differ when the DNA is digested with that restriction enzyme. The similarity of the patterns generated on an agarose gel can be used to differentiate species (and even strains) from one another (Viaud *et al.*, 2000).

1.4.2.4 DNA sequence analysis

DNA sequence analysis is currently the most commonly applied technique in molecular identification. Often allied to RAPD (Boysen *et al.*, 1996; Bulat *et al.*, 1998), or with RFLP (Gams & Meyer, 1998; Hambleton *et al.*, 1998; Viaud *et al.*, 2000), the fungal genomic (nuclear) DNA most frequently sequenced for the purposes of species identification is within the region of the genes for the ribosomes (Fig 1.3). Because nuclear ribosomal DNA (nrDNA) occurs as multiple repeats within the genome, typically present in a hundred or more copies per haploid genome (Metzenberg, 1991), extraction of sufficient material for analysis can be relatively straightforward. The nuclear small (18S) and large (28S) nrDNA subunits have evolved relatively slowly over time and sequence data can be useful in identifying distantly related organisms, generally to the family or genus level (O'Donnell *et al.*, 1997). On the other hand, the internal transcribed spacer sequences (ITS 1 and ITS 2; Fig 1.3) are under less stringent selection than the

nrDNA and so accumulate mutations more rapidly which can can give rise to variations among species within a genus or among populations (White *et al.*, 1990).

The ITS region, in its parts or overall, has proved particularly popular in phylogenetic analyses owing to this high level of variability in the nucleotide sequence which has allowed identification to be made to species level and to even differentiate between strains. A check of the National Centre for Biotechnology Information nucleotide data base (http://www.ncbi.nih.gov/Entrez/) indicates that there are some 20,000 fungal ITS sequences (including yeasts) currently lodged and available.

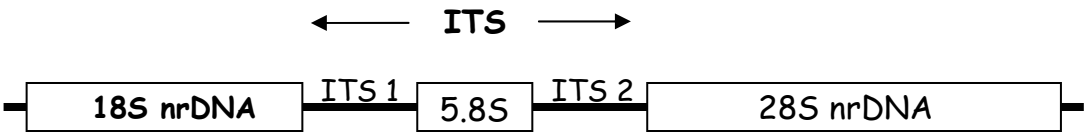

Figure 1.3 The arrangement of the ribosomal DNA showing the relative position of the small subunit (18S), large subunit (28S) and the internal transcribed spacer region (ITS)
The ITS region encompasses the variable ITS 1 and ITS 2 internal transcribed spacers and the conserved 5.8S nrDNA subunit.

1.5 Physiological factors affecting Antarctic microfungi

It has already been noted in 1.1 above that the Antarctic represents one of the most hostile environments for life on Earth. The terrestrial continental Antarctic is characterised by high stress/ high disturbance conditions that are exemplified by low temperatures, high aridity and a high incidence of uv radiation (Abele & Pörtner, 1998; Zucconi *et al.*, 1996). In order to adjust cellular metabolism, growth and development to environmental alterations, living cells employ specific sensory and signalling systems.

Any disturbance of the normal growth condition, even any deviation from optimal growth circumstances can be considered as stress imposed on cells (Mager & De Kruijff, 1995).

1.5.1 Stresses encountered by Antarctic microfungi

In the marine environment at the Casey station, mean temperatures are reported to range from just above freezing to -20ºC for the warmest and coldest months with extreme temperatures ranging from +9ºC to -41ºC (Azmi & Seppelt, 1997; 1998; Jackson & Seppelt, 1995). The climate is dry with an average annual snowfall of 195 mm per year (rainfall equivalent) and gale force winds, predominantly off the ocean, blow on average for 96 days in the year (Azmi & Seppelt, 1998). Whilst fungi usually grow best over a pH range of 5-7 (Azmi & Seppelt, 1997), the pH of ornithologically influenced soils in the neighbouring Windmill Islands was found to range from 4.6-8.5 (Azmi & Seppelt, 1997).

Away from the coastal influences conditions can be even more extreme. The McMurdo Dry Valleys are reportedly the most extreme cold habitat on Earth, multiply stressed by low temperature, aridity, high mineral-salt content, low substrate-carbon content, and very dubious photosynthetic productivity (Vishniac, 1993). The Dry Valley desert soils are generally poorly developed, coarse textured, and low in biological activity (Campbell and Claridge 1987, Campbell et al. 1998) and are unique among desert soils in having large amounts of soluble salts, high pH, and permafrost at 10-30 cm depth (Pastor and Bockheim 1980, Bockheim 1997). Surface soil (0-20 cm depth) temperatures in December and January are consistently above freezing, permitting biological activity (Vincent, 1989). For approximately 2-3 months in the austral summer, the dry valley climate is characterised by continuous daylight, near-freezing air temperatures, and the presence of meltwater flow from glaciers. During the seven months of winter, darkness is nearly continuous, surface water is absent, and air temperatures on the valley floor fall to

as low as -40ºC (Clow *et al.*, 1988). The very low amount of precipitation mainly falls as snow, although small amounts of rain have been known to occur during the summer (Keys, 1980). Average annual precipitation (water equivalent) in the valleys is less than 100 mm (Fountain *et al.*, 1999).

The overall impact of these extremely hostile conditions is to subject the resident microfungi to high levels of stress. The universally low availability of liquid water and the desiccating conditions, particularly in the Dry Valleys region, brought about by constant wind (Clow *et al.*, 1988), subject each organism to a greater or lesser level of osmotic stress. This stress factor can be further aggravated by the high salt and pH levels present in many environments, a result of maritime and ornithogenic influences or inherent in the soils. Notwithstanding the protection that may be offered by surrounding soil layers or via an association with microflora (bryophytes or algae), fungi can be further stressed by the extreme temperature fluctuations between day and night, either diurnal and/or seasonal, which can result in repetitive freeze/thaw cycles. Additionally, the fungi can be subjected to oxidative stress, a result of other prevailing stress factors (Hazell *et al.*, 1997), due to the high incidence of uv radiation (Abele & Pörtner, 1998; Moradas-Ferreira *et al.*, 1996) or inherent in its own metabolic processes (Jamieson, 1998; Joseph-Horne *et al.*, 2001; Kreiner *et al.*, 2002; Møller *et al.*, 1996; Moradas-Ferreira *et al.*, 1996; Osiewacz, 2002). To safeguard itself against these exigencies, each organism has had to acquire a protective strategy to ensure its continued survival.

1.5.2 Stress reponses by Antarctic microfungi

Any disturbance to the normal growth conditions including deviations from optimal growth circumstances can be viewed as a stress imposed on a cell (Mager & De Kruijff, 1995). Such conditions, either physical or chemical, that impose a negative effect on

maximum growth will result in cellular responses that ensure at best continued growth of the microfungi or at worst survival until favourable growth conditions are again established. To protect cells against the detrimental effects that stress may impart in the short and long term, stress response mechanisms act to repair any immediate damage inflicted on the cell. Consequently, stress response can result in adjustments to metabolic activity and other cellular processes to ensure either growth and/or survival in the altered environment. Stress treatment and stress response may lead to the synthesis of new proteins, a cessation of growth, alterations in membrane composition and the production of metabolites such as trehalose, glycerol or glutathione. Although the type of proteins, enzymes or metabolites may vary for a particular stress applied, the overall aim of stress responses is thought to be to maintain an environment that retains biological function and ultimately will result in survival in the long term under stress conditions (Mager & Hohmann, 1997).

Unfortunately, there is a paucity of literature dealing with stress responses in filamentous fungi and virtually none specific to Antarctic microfungi. In order to gain some insight into the likely mechanisms which govern stress response in Antarctic microfungi it was necessary to draw on the more extensive literature available for microorganisms at large, and for yeast in particular. As a large component of the yeast literature dealing with stress response is concentrated upon the industrial yeast strains, notably brewing and baker's yeast (*Saccharomyces cerevisiae*), any extrapolation of the mechanisms employed by yeast to Antarctic microfungi must be circumspect at the least.

1.5.3 Cross protection in response to stress

The complexity of stress tolerance is shown in cross-protection or acquisition of tolerance to a particular stress by an apparently unrelated mild stress treatment. It has been

observed that exposure to a single stress condition can lead to an increased tolerance to other unrelated stressful conditions suggesting that there may be a common basis for stress response (Hazell *et al.*, 1997). In the case of *Saccharomyces cerevisiae*, heat-shock can protect against freezing, and heat tolerance can be obtained by osmotic stress and vice-versa (Lewis *et al.*, 1995; Trollmo *et al.*, 1988; Varela *et al.*, 1992). Heat stress conditions have been shown to induce a general response, in common with other metabolically adverse circumstances leading to physiological perturbations, such as oxidative stress or osmostress (Mager & De Kruijff, 1995). The concept of a general stress response suggests that there are shared mechanisms or signalling pathways (Fillinger *et al.*, 2001; Siderious & Mager, 1997) irrespective of the condition causing cellular stress. Stress responsive genes and products have been categorised by where they were first observed following a particular stress treatment. In yeast, heat shock proteins and trehalose accumulation are associated with heat-shock; osmotically inducible proteins and glycerol are associated with osmotic shock; glutathione, antioxidant enzymes and metabolites are associated with oxidative shock treatment (Mager & De Kruijff, 1995).

1.5.4 Trehalose and compatible solutes

Trehalose is a disaccharide that is ubiquitous in the biosphere. It consists of two subunits of glucose bound by an α:1→1 linkage (α-D –glucopyranosyl α-D –glucopyranoside) and is thus nonreducing. Trehalose has been isolated and characterised from a wide variety of organisms including yeasts and filamentous fungi (Argüelles, 2000; Elbein, 1974; Fillinger *et al.*, 2001; Strom & Kaasen, 1993; Thevelein, 1984; van Dijck *et al.*, 1995) and is often present in large quantities in cells where severe water shortages may occur during their life cycle. The success of trehalose in nature as a stress metabolite results from a number of unique physico-chemical properties, which include high hydrophilicity

and chemical stability, non-hygroscopic glass formation and the absence of internal bond formation (Argüelles, 2000; Thevelein, 1996).

1.5.4.1 Trehalose as a storage carbohydrate

Studies in both yeasts and filamentous fungi have suggested that trehalose also constitutes a major reserve compound although this function has given rise to considerable controversy (Van Laere, 1989; Wiemken, 1990). The universal mobilization of trehalose during growth resumption from resting stages has suggested this role as a storage carbohydrate (Argüelles, 2000; Elbein, 1974; Thevelein, 1984; Thevelein, 1996). Other evidence suggesting that trehalose acts as a storage carbohydrate is that the metabolites glucose-6-phosphate and UDP-glucose are used to synthesize both trehalose and glycogen, the latter being well established as an energy reserve (Fig 1.4). In the yeast *Saccharomyces cerevisiae*, biosynthesis of trehalose is mediated by a multi-protein

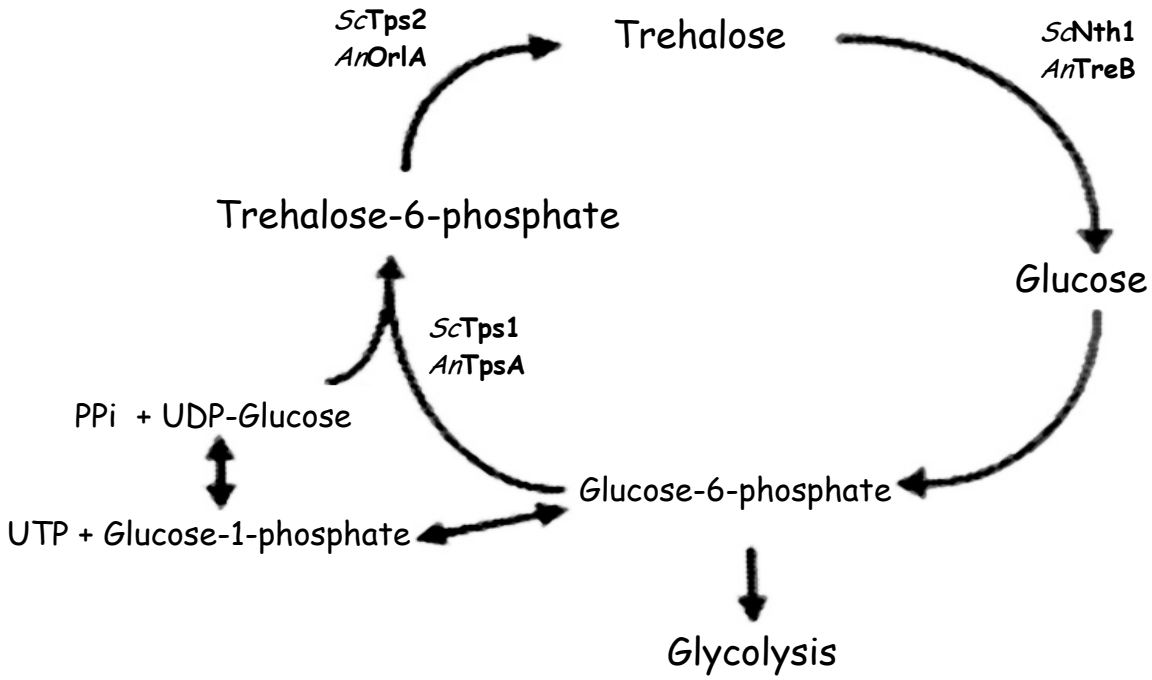

Figure 1.4 Trehalose metabolism in fungi. (Fillinger et al., 2001) *Sc*Tps*1* and *An*TpsA, trehalose-6-phosphate synthase; *Sc*Tps2 and *An*OrlA**,** trehalose-6-phosphate phosphatase; *Sc*Nth1 and *An*TreB, neutral trehalase; *Sc, Saccharomyces cerevisiae; An, Aspergillus nidulans.*

complex (Fillinger *et al.*, 2001) that contains a trehalose-6-phosphate synthase (Fig 1.4) encoded by *TPS1* and a trehalose-6-phosphate phosphatase (Fig 1.4) encoded by *TPS2*. Mobilisation of the trehalose pool in response to various stimuli is mediated by a neutral trehalase Nth1. Fillinger *et al.* (2001) have demonstrated that the filamentous microfungus *Aspergillus nidulans* sustains a similar protein complex (Fig 1.4).

1.5.4.2 The protective role of trehalose

There appears to be no controversy surrounding the protective role of trehalose in yeast and fungi as a stress metabolite (Argüelles, 2000; Fillinger *et al.*, 2001; Robinson, 2001; Thevelein, 1996). The protective action of trehalose is due to its unusual chemical properties which makes it resistant to heating, protects dehydrated cellular membranes (Crowe *et al.*, 1984) and increases the thermal stability of proteins (Hottiger *et al.*, 1994). On dehydration, trehalose forms a hydrophilic glass (as opposed to crystals formed by other sugars) which reduces distortion of macromolecules (membranes and proteins) and improves the likelihood that biological function will be retained upon rehydration. Accumulation of trehalose has been shown to be a crucial factor in the adaptive response and as a protectant during to a variety of environmental stress conditions (Thevelein, 1984; Van Laere, 1989; Wiemken, 1990) such as heat (De Virgilio *et al.*, 1994; Lewis *et al.*, 1995), freezing (De Virgilio *et al.*, 1994; Lewis *et al.*, 1995), dehydration and dessication (D'Amour *et al.*, 1991; Gadd *et al.*, 1987; Hottiger *et al.*, 1987) and exposure to toxic chemicals and oxidative agents (Alvarez-Peral *et al.*, 2002; Attfield, 1987; Fillinger *et al.*, 2001).

Under conditions of freezing- or drying-induced dehydration, trehalose shows an exceptional capacity to protect enzymes and biological membranes (Argüelles, 2000). Trehalose has also been identified in a broad range polar biota to act as an effective

cryoprotectant (Montiel, 2000). In addition, the rapid hydrolysis of trehalose by trehalase (Fig 1.4), is necessary during stress recovery for cellular structures to be liberated from bound carbohydrate (Singer & Lindquist, 1998). Industrially, trehalose has been found to increase the stability of several unstable commodities during dry storage or freezing. By virtue of this ability, it has become a widely valued preservative for such products as enzymes, foods, pharmaceuticals and cosmetics (Colaço *et al.*, 1992).

1.5.4.3 Glycerol as a compatible solute

When the external cell environment increases in osmotic pressure to the point where cell contents are more dilute, water is lost to the external surroundings. To regain the lost water, the cell increases its internal osmotic pressure by accumulating compatible solutes (Blomberg & Adler, 1992). There is extensive evidence that polyols play an important role in generating the internal osmotic potential when fungi are grown in media of decreasing water potential (Jennings & Burke, 1990). Glycerol, mannitol and sorbitol have each been recognised as compatible solutes in fungi (Jennings & Burke, 1990) as have other substrates such as the amino acid proline and the trimethylammonium compound glycine betaine (Kempf & Bremer, 1998; Popova & Busheva, 2001).

Glycerol appears to be one of the principal compatible solutes employed by yeasts and fungi in response to osmotic shock. *S. cerevisiae* produces glycerol under hyperosmotic stress and genetic analysis has confirmed that the glycerol accumulation is a determinant of tolerance to osmotic stress (Hazell, 1999). Recent investigations with the biocontrol fungus, *Penicillium frequentans*, have demonstrated that glycerol was the main compatible solute accumulated at reduced water potential (Pascual *et al.*, 2000). The filamentous yeast, *Ashbya gossypii*, was also found to have predominantly accumulated

glycerol as a compatible solute when subjected to hyperosmotic stress (Foerster *et al.*, 1998).

In conditions of increased external osmolarity which may arise due to desiccating conditions, elevated levels of salt or pH, yeasts have been shown to experience a sudden reduction in cell volume from the loss of water to the external environment (Albertyn *et al.*, 1994). To regain the lost water, yeasts will produce and retain glycerol in the cytoplasm. The production and retention of glycerol increases the internal osmolarity of the cytosol with minimal impact to biochemical processes occurring in the cell (Hazell, 1999). Glycerol is retained in the cell until the hyperosmotic stress is relieved (Hohmann, 1997; Luyten *et al.*, 1995).

1.5.5 Oxidative stress

All aerobically growing organisms suffer exposure to oxidative stress, caused by partially reduced forms of molecular oxygen, know as reactive oxygen species (Emri *et al.*, 1997; Jamieson, 1998; Kreiner *et al.*, 2002). Reactive oxygen species (ROS) include singlet oxygen ($^1O_2$), the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH^-) and these have been shown to be highly damaging towards cellular constituents including DNA, lipids and proteins (Alvarez-Peral *et al.*, 2002; Jamieson, 1998; Moradas-Ferreira *et al.*, 1996; Santoro & Thiele, 1997). Although the superoxide radical and H_2O_2 do not react directly with DNA, OH^- is generated from them by the metal-catalysed Fenton/Haber-reaction. A detailed explanation of the reactions involved in oxygen activation and the biological reactions of oxygen radicals is provided by McKersie (1996) in his on line presentation at http://www.plantstress.com/Articles/Oxidative Stress.htm.

Free oxygen radicals and other ROS are formed as by-products in normal metabolism, a result of the 'leakage' of electrons from the cellular (mitochondrial) electron transport chain. They may also be generated for metabolic and physiologic functions in specialized cells or by H_2O_2-generating reactions catalysed by oxidases (Jamieson, 1998; Kreiner *et al.*, 2002; Møller *et al.*, 1996; Moradas-Ferreira *et al.*, 1996). ROS can also be produced as part of a reaction to environmental toxic substances (redox-cycling chemicals, heavy metals) and by ionising radiation (Jamieson, 1998; Møller *et al.*, 1996; Moradas-Ferreira *et al.*, 1996). It has also been shown that singlet oxygen is generated in *Neurospora crassa* during germination of conidia (Lledías *et al.*, 1999).

As a defence against damage induced by ROS, aerobically growing organisms have evolved a range of strategies. These strategies include defensive enzymes, such as superoxide dismutases, catalases and peroxidases as well as non-enzymatic antioxidants such as ascorbate and tocopherols (Kreiner *et al.*, 2002). Under normal physiological conditions, the toxic effects of ROS are minimised by these enzymatic and non-enzymatic antioxidants. Under stressful conditions, such as microfungi would experience in the Antarctic, when reactive oxidants increase beyond the antioxidant buffering levels, oxidative stress occurs.

Although the oxidative stress response which protects organisms from the deleterious effects of ROS has been extensively examined in bacteria and yeast, surprisingly little data are available on the antioxidative defence systems of filamentous fungi (Kreiner *et al.*, 2002). With the yeast *Saccharomyces cerevisiae*, fluorescent probes have been utilised in an attempt at estimating the level of oxidative stress present (Jakubowski & Bartosz, 1997). The use of fluorogenic substrates alone can at best provide only a qualitative assessment of the level of enzymatic activity occurring in the cell. A technique

which has been utilised extensively with yeast to enumerate various cell properties is flow cytometry (Bell *et al.*, 1998; Malacrinò *et al.*, 2001) which could, in conjunction with suitable fluorescent stains, provide a quantitative evaluation of oxidative stress response. To date, the use of flow cytometry has not been extended to the measurement of enzymatic activity in developing filamentous microfungi.

1.6 Flow cytometry and microfungi

Isolation, detection and most routine analyses of microfungi use culture based methodologies: solid medium on agar plates or in liquid medium with shake flasks. These methods are generally slow, taking from a couple of days to weeks to obtain results. Molecular techniques, based on PCR, can produce results within hours and with 'clean' samples can provide very sensitive methods for detection. However, their general application is limited and not suitable for the isolation of a whole organism. The application of flow cytometry represents a technique which has the potential to overcome the above limitations and provide a powerful tool to advance the study of Antarctic microfungi.

1.6.1 Instrumentation

Flow cytometers are used to quantitatively measure the optical characteristics of particles, such as cells, as they are presented in single file, into a focused light beam. Flow cytometry is a technique for making rapid measurements on individual particles, at rates of 40,000 or more per second, as they are carried past a sensing point within a fast flowing fluid stream termed the sheath flow (Deere *et al.*, 1996). The important feature of flow cytometric analysis is that measurements are made separately on each particle, and are not an average value for the whole population.

Modern laser based flow cytometers are capable of measuring multiple cellular parameters simultaneously based on light scatter and fluorescence. A laser light beam hitting a stream of cells is scattered in all directions and detectors, with the aid of photomultipliers (PMT), measure forward angle light scatter (FSC), 90º or side scatter (SSC) and fluorescence (FL) (Fig 1.5). FSC detection can be viewed as an approximation of the size of a cell or particle passing the detectors and SSC an approximation to granularity or shape (Shapiro, 1995). When the laser beam excites a fluorochrome, the emitted light is detected by three (sometimes more) fluorescence detectors.

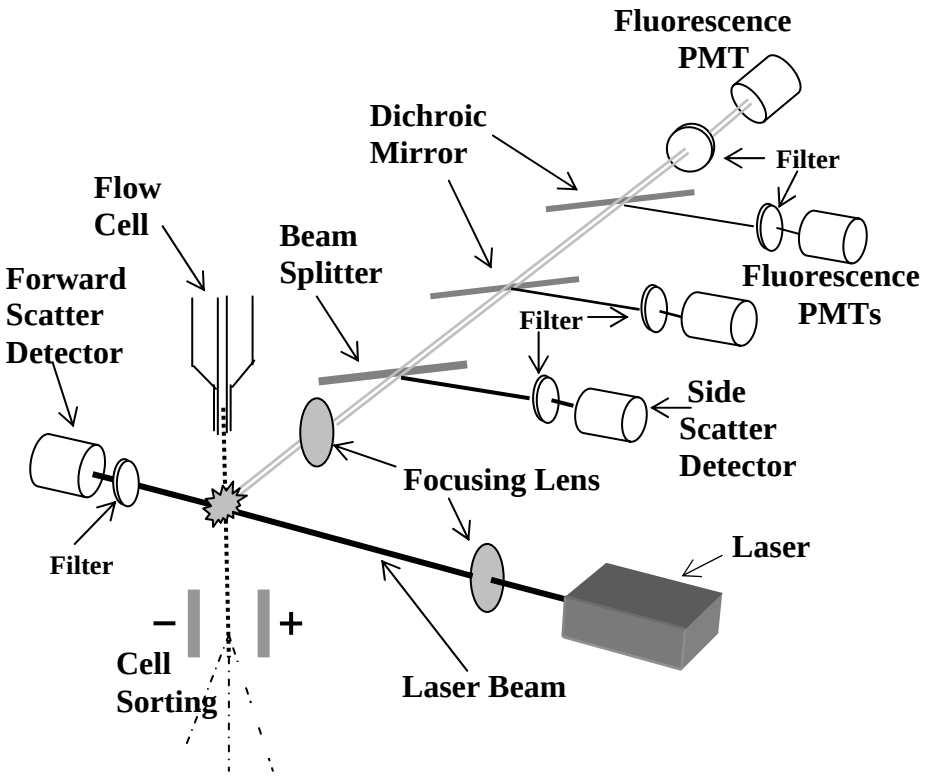

Figure 1.5 Schematic of a flow cytometer

An additional feature available on many modern flow cytometers is the ability to separate (sort) cells specifically identified during analysis (Omerod, 2000; Vives-Rego *et al.*, 2000). Any combination of parameters can be applied as criterion to decide whether a cell is or is not sorted and those selected can then be directed (collected) to optical slides

or a suitable membrane for further analysis. Of particular importance for ongoing research is the non-destructive nature of the sort capability; organisms which are viable when introduced to the flow cytometer invariably remain viable after sorting.

1.6.2 Application of flow cytometry

The use of flow cytometry has been widely documented in its application to bacteria (Bernander *et al.*, 1998; Katsuragi & Tani, 2000; Malacrinò *et al.*, 2001; Veal *et al.*, 2000; Vives-Rego *et al.*, 2000), to yeast (Bell *et al.*, 1998; Malacrinò *et al.*, 2001) and to fungal spores (Carr & Shearer, 1998; De Lucas *et al.*, 1998; Gourmet *et al.*, 1997; Kim *et al.*, 2001; Smith *et al.*, 1999). Although Hopfer *et al* (2001) reported on filament formation in *Candida albicans* and briefly examined filamentation in heat killed *Aspergillus fumigatus*, virtually no work has been documented on the application of flow cytometry to filamentous fungi in the early growth stages when hyphae have started to develop apart from some preliminary investigations described by Nevalainen (2001).

A wide range of fluorescent probes are available (Hagerman *et al.*, 1985) which will permit the direct detection of a variety of properties, such as amounts of various cell components, specific sequences of peptides and nucleotides, cell functions and enzyme activities (Bernander *et al.*, 1998; Fitzpatrick *et al.*, 2000; Katsuragi & Tani, 2000; Omerod, 2000; Reiseberg *et al.*, 2001). Some fluorochromes (fluorescent dyes) can penetrate the cell membrane and enter the cytosol whilst others can be attached to antibodies that are specific to cell surface markers or can enter the cell and interact with the cell's metabolic processes (Haugland, 1996). Natural fluorescence (autofluorescence) and fluorescent substrates can be utilised to elicit information on individual cells, cellular components and metabolic activity.

1.7 Hydrolytic enzymes of industrial interest

There are nearly 4000 enzymes known today and of these about 200 are in commercial use (Sharma *et al.*, 2001). The majority of the industrial enzymes are of microbial origin. Hydrolases define that group of enzymes which catalyse the hydrolysis of substrates (Fieser & Fieser, 1956; McMurry, 1992) and include among them carbohydrases (amylases, cellulases and hemicellulases), esterases (lipases) and proteases. At least 75% of all industrial enzymes are hydrolytic in action, being used for the degradation of various natural substances (Godfrey & West, 1996).

Since ancient times, enzymes found in nature have been used in the preparation of food products, such as, beer, wine, vinegar and cheese, and in the manufacture of commodities such as leather, indigo and linen (Kirk *et al.*, 2002). Today, enzymes produced by fermentation of microorganisms, represent approximately 90% of all enzymes applied in industrial processing and fungi have become an essential part of this operation (Godfrey & West, 1996).

Most of the enzymes produced today for industrial applications have been isolated from mesophiles and thermophiles. In the case of mesophiles, this stems from the wealth of material available and ease of acquisition from environmental sources and for thermophiles, due to their thermostability for many industrial processes (Ohgiya *et al.*, 1999). Table 1.2 shows the major industrial segments utilising enzymes and their application (from Kirk *et al.*, 2002) and also shows the fungal sources of these enzymes (from Godfrey & West, 1996). The value of industrial enzymes used worldwide was in the vicinity of US$1 billion in 1995 (Godfrey & West, 1996) and had grown to an estimated US$1.5 billion by 2000 (McCoy, 2000). Some 40% of this production is

believed to have been derived from fungal sources (M. G. Weibe at http://www.bio.auc.dk/~mgw/Industrial Microbiology/5a Fungal enzymes.pdf/).

1.7.1 Cold adapted enzymes – structure and function

As all filamentous fungi acquire their sustenance via the secretion of enzymes into their environment (Johnson & Raven, 2002), it can reasonably be surmised that Antarctic microfungi have acquired adaptations to many of their enzymes sufficient to allow them to remain functional in the face of the intense cold. Although significant progress has been made in elucidating the molecular adaptation mechanism of enzymes produced by extremophiles such as hyperthermophiles, the molecular basis of cold adaptation is still relatively poorly understood. However, data are now being accumulated on psychrophilic enzymes which is starting to shed some light on their functional and structural characteristics (Feller *et al.*, 1997; Gianese *et al.*, 2001). Enzymes produced by cold adapted microorganisms most commonly display a high catalytic efficiency at low temperatures measured as k_{cat}/K_M and a significantly increased thermolability believed to be a consequence of enhanced peptide chain flexibility. Using X-ray crystallography, these properties are beginning to become understood and the rules governing their adaptation to cold appear to be relatively diverse.

Gianese *et al* (2001) have carried out a comparative analysis of 21 psychrophilic enzymes belonging to different structural families from prokaryotic and eukaryotic organisms. The sequences of these enzymes were multiply aligned to 427 homologous proteins from mesophiles and thermophiles. Homology modelling was utilised to predict the secondary structure and accessibility of amino acid residues for the psychrophilic enzymes. The results obtained showed a clear tendency for the charged residues Arg and Glu in “hot” enzymes to be replaced at exposed sites on α-helices by Lys and Ala

Table 1.2 **Enzymes used in various industrial segments, their applications and fungal sources**
(Adapted from Kirk *et al.* (2002) and Godfrey & West (1996)).

Industry	Enzyme class	Application	Source
Detergent (laundry & dish wash)	Protease	Protein stain removal	1,2,6,7 & 10
	Amylase	Starch stain removal	1,6 & 11
	Lipase	Lipid stain removal	1,2,5,6 & 7
	Cellulase	Cleaning, colour clarification, anti-redeposition (cotton)	1,2,3 & 4
	Mannanase	Mannanan stain removal (reappearing stains)	1,3,4 & 12
Starch and fuel	Amylase	Starch liquefaction and saccharafaction	1,6 & 11
	Amylo-glucosidase	Saccharafaction	1
	Pullulanase	Saccharafaction	N
	Glucose isomerase	Glucose to fructose conversion	N
	Cyclodextrin--glycosyltransferase	Cyclodextrin production	N
	Xylanase	Viscosity reduction (fuel & starch)	1,3,4 & 12
	Protease	Protease (yeast nutrition – fuel)	1,2,6,7 & 10
Food (including dairy)	Protease	Milk clotting, flavour, infant formulas (low allergenic)	1,2,6,7 & 10
	Lipase	Cheese flavour	1,2,5,6 & 7
	Lactase	Lactose removal (milk)	1,8 & 9
	Pectin methyl esterase	Firming fruit-based products	1,3 & 4
	Pectinase	Fruit-based products	1,3 & 4
	Transglutaminase	Modify visco-elastic properties	np
Baking	Amylase	Bread softness and volume,	1,6 & 11
	Xylanase	Dough conditioning	1,3,4 & 12
	Lipase (*in situ* emulsifier)	Dough stability and conditioning	1,2,5,6 & 7

continues

Table 1.2 *continued*

Baking (continued)	Phospholipase	Dough stability and conditioning (*in situ* emulsifier)	1
	Glucose oxidase	Dough strengthening	1 & 2
	Oxygenase	Dough strengthening, bread whitening	N
	Protease	Biscuits, cookies	1,2,6,7 & 10
	Transglutaminase	Laminated dough strengths	np
Animal feed	Phytase	Phytate digestibility- phosphorous release	1
	Xylanase	Digestibility	1,3,4 & 12
	β-Glucanase	Digestibility	1,2,3,4 & 8
Beverage	Pectinase	Depectinisation, mashing	1,3 & 4
	Amylase	Juice treatment, low calorie beer	1,6 & 11
	β-Glucanase	Mashing	1,2,3,4 & 8
	Acetolactate decarboxylase	Maturation (beer)	N
	Laccase	Clarification (juice), flavour (beer), cork stopper treatment	N
Textile	Cellulase	Denim finishing, cotton softening	1,2,3 & 4
	Amylase	Desizing	1,6 & 11
	Pectin lyase	Scouring	1,3 & 4
	Catalase	Bleach termination	1 & 2
	Laccase	Bleaching	N
	Peroxidase	Excess dye removal	1 & 2
Pulp and paper	Lipase	Pitch control, contaminant control	1,2,5,6 & 7
	Protease	Biofilm removal	1,2,6,7 & 10
	Amylase	Starch-coating, deinking, drainage improvement	1,6 & 11
	Xylanase	Bleach boosting	1,3,4 & 12
	Cellulase	Deinking, drainage improvement, fibre modification	1,2,3 & 4

continues

Table 1.2 *continued*

Fats and oils	Lipase	Transesterification	1,2,5,6 & 7
	Phospholipase	Degumming, lyso-lecithin production	1
Organic synthesis	Lipase	Resolution of chiral alcohols and amides	1,2,5,6 & 7
	Acylase	Synthesis of semisynthetic penicillin	1
	Nitrilase	Synthesis of enantiopure carboxylic acids	np
Leather	Protease	Unhairing, bating	1,2,6,7 & 10
	Lipase	Depickling	1,2,5,6 & 7
Personal care	Amyloglucosidase	Antimicrobial (combined with glucose oxidase)	1 & 6
	Glucose oxidase	Bleaching, antimicrobial	1 & 2
	Peroxidase	Antimicrobial	1 & 2

Fungal Sources

1. *Aspergillus* spp.
2. *Penicillium* spp.
3. *Trichoderma* spp.
4. *Humicola* spp.
5. *Candida* spp.
6. *Rhizopus* spp.
7. *Mucor* spp.
8. *Saccharomyces* spp.
9. *Kluyveromyces* spp.
10. *Endothia parasitica*
11. *Endomyces* spp.
12. *Trametes* spp.

N No fungal source
np Source not provided

(respectively) in their “cold” analogues. At buried regions in α-helices, Val is replaced by Ala. Compositional analysis of psychrophilic enzymes shows significant increase of Ala and Asn and a decrease of Arg at exposed sites. Buried sites in β-strands tend to be depleted of Val.

The preferred amino acid exchanges observed from thermo/mesophiles to psychrophiles and compositional analysis indicate a decreased number of side chain potential H-bonds and salt bridges in cold-adapted enzymes. Rigid secondary structures and disulfide bridges are practically absent, thus accounting for increased thermolability. Cold adaptation strategy seems to apply the reverse principle to that of hot adaptation, namely a decrease in the number of H-bonds/ion pairs as you go from high to low temperatures (Feller & Gerday, 1997; Feller *et al.*, 1997; Marshall, 1997a). The tertiary and quaternary structures of cold-active enzymes appear to have more open and flexible arrangements, thus providing better access of substrates to the active site at lower temperatures (Nichols *et al.*, 2002). Individual enzyme types possess different structural strategies to gain overall increased flexibility. Certain structural features thought to be indicative of cold adaptation have also been found in similar non-cold adapted enzymes (Schroder *et al.*, 1998). In addition, the same enzyme from different organisms, containing an identical amino acid sequence, has been found to possess different thermal properties (Love & Marshall, 1998). This suggests that protein folding has a critical role in conferring activity at low temperature.

Just as enzymes from thermophiles often have optimal activity at temperatures higher than found for mesophilic organisms, so cold-active enzymes shift their peak activities to temperature ranges lower than those generally observed for mesophilic enzymes

(Brenchley, 1996). Enzymes isolated from cold-adapted microorganisms can be classified into three groups (Fig 1.6) (Ohgiya *et al.*, 1999):

Group I: Heat-sensitive. Other enzymatic characteristics are similar to mesophilic enzymes.

Group II: Heat-sensitive and relatively more active than mesophilic enzymes at low temperature.

Group III: Same thermostability as mesophilic enzymes but more active than mesophilic enzymes at a low temperature.

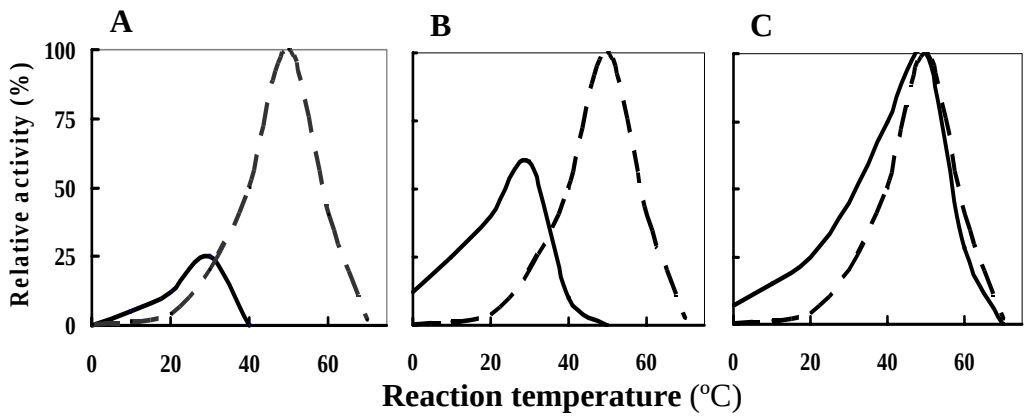

Figure 1.6 Three types of enzymes isolated from cold-adapted microorganisms
(From Ohgiya *et al.*, 1999)
Enzymes from cold-adapted microorganisms
Enzymes from meso- or thermophilic microorganisms
A, Group I; B, Group II, C, Group III.

The specific activity of a number of cold-adapted enzymes produced by Antarctic and Arctic microorganisms (wild-type and recombinant forms) has been determined (Gerday *et al.*, 2000). Typically, the specific activity of these enzymes is higher than that of their mesophilic counterparts at temperatures of approximately 0 – 30ºC. At higher temperatures, denaturation of the cold-active enzyme can be expected to occur.

1.7.2 Industrial application of cold-adapted enzymes

There is a growing interest in cold adapted enzymes due to their potential in industrial applications. Psychrophilic enzymes produced by cold-adapted microorganisms display a high catalytic efficiency that offers considerable potential to the biotechnology industry, making it possible to reduce the amount of enzyme needed in catalytic processes (Demirjian *et al.*, 2001). The low temperature optimum and temperature stability makes them preferable for use in reactions performed at low temperatures that needs an easy inactivation (Yamamura *et al.*, 1999). From the biotechnological point of view cold-adapted enzymes, which antarctic microfungi can potentially supply, could in the future replace their mesophilic counterparts and help to establish new improved bioprocesses under low temperature conditions. These enzymes can help to save energy, to save labile or volatile compounds, to prevent the growth of mesophilic contaminants at low temperature, and could be easily inactivated by moderate temperatures.

The cold-adapted enzymes cover a broad spectrum of biotechnological applications (Feller *et al.*, 1996; Gerday *et al.*, 2000; Marshall, 1997a; Yamamura *et al.*, 1999). Some of the potential advantages of using cold-active enzymes in industrial processes are listed in Table 1.3.

Antarctic microfungi are likely to be a useful source of biotechnologically useful cold-adapted enzymes. Fungi can be screened to identify naturally occurring cold adapted enzymes displaying potentially useful properties.

Table 1.3 Industrial applications of enzymes at low temperatures

(From Ohgiya *et al.*, 1999)

Application	Enzymes	Advantages
Detergent	protease, lipase, cellulase, etc.	use in tap water
Food industries:		
Modification of constituents	galactosidase, lipase	keeping freshness
Improvement of taste & flavour	protease, lipase, etc.	keeping freshness
Removal of fish skin	protease	product quality
Clarification of fruit juice	pectinase, cellulase	keeping fragrance
Preservation	lysozyme, glucose oxidase	improved preservative effect
Enzymatic synthesis	lipase, nirile hydratase, etc	for volatile and heat sensitive enzymes
Treatment of waste water	catalase	lower energy consumption
Biotechnology research:		
Protoplast formation	cell wall digesting enzymes	high viability
Molecular biology	phosphatase, uracil DNA glycosylase	complete heat inactivation

1.7.3 Screening for enzyme activity

All fungi secrete enzymes into their environment in order to reduce complex biological molecules into basic units which are then absorbed back into the fungus as nutrients (Johnson & Raven, 2002). If the carbon sources available to a fungus are restricted, it is induced, where possible, to secrete enzymes suitable for their mineralisation for its very survival. This requirement of fungi to secrete enzymes can be harnessed to provide a relatively simple method of screening for the presence of a targeted enzyme.

By the simple strategy of culturing fungi on agar plates containing a specific carbon source or an additive in the growth medium, the presence of a targeted enzyme can be identified by its known reaction in the presence of the substrate(s). This method of screening on solid media has been progressively developed over the last 30 odd years. It is used extensively to identify the absence or presence of specific enzymes in fungi and as a means to rapidly search for genetic variants. The broad use of solid media to detect

fungal enzyme production was systematised by Hankin and Anagnostakis (1975) who developed diagnostic media for the identification of nine types of enzymatic activity (Table 1.4). The range of enzymes that can now be screened on solid media has been extended through the work of many investigators. Table 1.4 illustrates some examples of the enzymes that can be targeted and the additives that are included in the growth media.

Actual detection of the enzymatic activity can sometimes be accomplished directly due to the appearance of clearing zones surrounding positive colonies, e.g. proteases (Fig 1.7a) or by fluorescence when viewed under uv light as with lipase activity and rhodamine B.

Table 1.4 Additives to solid media to detect enzymatic activity

Enzyme activity	Additive	References
Amylase	soluble starch	Hankin & Anagnostakis, 1975
Cellulase	Walseth cellulose	Walseth, 1952 Nevalainen, 1985
	carboxymethyl cellulose	Melo *et al.*, 1997
	(hydroxyethyl cellulose)	Nevalainen, 1985 Hagerman *et al.*, 1985
Chitinase	chitin	O'Brien & Colwell, 1987 Hankin & Anagnostakis, 1975
DNAse	deoxyribonucleic acid	Hankin & Anagnostakis, 1975
β-glucanase	AZCL-pachyman	Appendix I
β-glucosidase	Esculin [1] & FAC [2]	Melo *et al.*, 1997
	cellobiose	Nevalainen, 1985
Lipase	sodium monolaurate (Tween 20)	Hankin & Anagnostakis, 1975
	olive oil & rhodamine B	Kouker & Jaeger, 1987
β-mannanase	locust bean gum	Rättö & Poutanen, 1988
	OBR-galactomannan [3]	Kremnický *et al.*, 1996
Pectinase	pectin (citrus or apple)	Hankin & Anagnostakis, 1975 Hagerman *et al.*, 1985
Phosphatase	phenolphthalein diphosphate (sodium salt)	Hankin & Anagnostakis, 1975
Protease	gelatin	Hankin & Anagnostakis, 1975 Hagerman *et al.*, 1985
	skim milk	Mäntylä *et al.*, 1994
RNAse	ribonucleic acid (yeast)	Hankin & Anagnostakis, 1975
Urease	urea	Hankin & Anagnostakis, 1975
Xylanase	birch xylan	Appendix I
	RRB-xylan [4]	Kremnický *et al.*, 1996

[1] Esculin – 7,6-dihydro-oxycoumarin-6-glucoside
[2] FAC – ferric ammonium citrate
[3] OBR-galactomannan – Ostazin Brilliant Red-gallactoglucomannan
[4] RRB-xylan – Remazol Brilliant Blue-xylan

For other enzymes, detection may require secondary reactions to identify the activity. A stain/destain step to is required to visualise activity for β-mannanase (locust bean gum), xylanase (birch xylan) and cellulase (carboxymethyl cellulose or other soluble cellulose). When stained with Congo Red, these plates will display a clearing zone or halo when destained with NaCl (Fig 1.7c). Similarly, pectinase screening plates, when stained with ruthenium red and destained with water will produce either a clearing zone in the

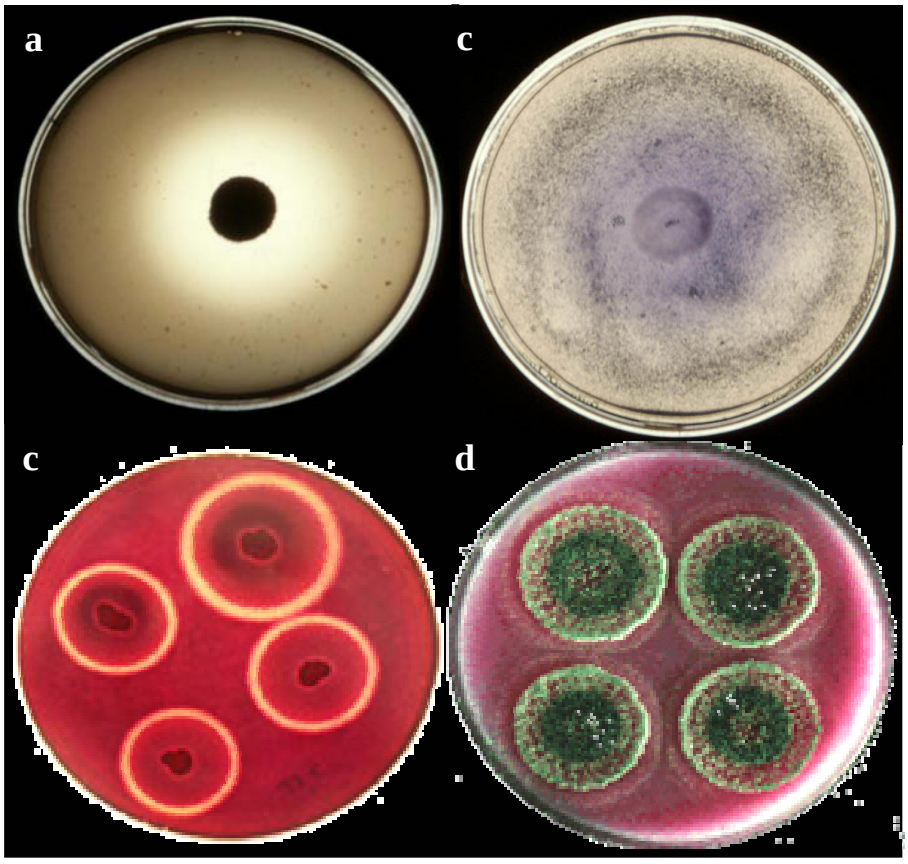

Figure 1.7 Detection of enzyme activity on solid media containing indicative carbon sources

a, *Penicillium commune* with skim milk. Clearing zone indicates protease activity.

b, *Trichoderma koningii* with AZCL-pachyman. Solubilization indicates β-glucanase activity.

c, *Embellisia* sp2 with locust bean gum. When stained with Congo Red a clear halo indicates mannanase activity.

d, *Trichoderma harzianum* with olive oil and rhodamine B. If irradiated by uv light, lipase activity would be indicated by orange fluorescence.

presence of pectin lyase or deeper colouration with pectin methyl esterase. When plates containing chitin are reacted with 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide (4-

MUF.GlcNAc) and viewed under uv light, the presence of chitinase can be identified by light-blue fluorescence. For other selection plate assays, various combinations of substrate solubilisation, clearing and colour change indicate the presence of enzymatic activity.

A range of products specifically designed to identify the presence of fungal (and other microorganisms') *endo*-hydrolase activity on culture plates are available commercially (see http://www.megazyme.com). These AZCL-Polysaccharides (Azurine-Crosslinked Polysaccharides) are prepared by dyeing and crosslinking highly purified polysaccharides. They are supplied as a fine powder which rapidly hydrates to form gel particles which are readily and rapidly hydrolysed by specific *endo*-hydrolases releasing soluble dye-labelled fragments. In the presence of the hydrolysing enzyme the dyed particles are observed to solubilise in the basal plate medium (Fig 1.7b).

1.7.4 Hemicellulases

In nature, in addition to cellulases, ligninases and pectinases, the hemicellulolytic enzymes are responsible for converting structural plant polymers to soluble monomers. The xylanases and mannanases constitute the two main hemicellulases responsible for the degradation of hemicelluloses.

1.7.4.1 The nature of hemicelluloses

Hemicelluloses are the second most abundant polysaccharide in nature after cellulose. Whereas cellulose has a homopolysaccharide structure where all the polysaccharide units are the same, hemicellulose has a hetropolysaccharide make-up. The term hemicellulose is used to refer to a wide variety of heteropolysaccharides found in association with cellulose and lignin in both woody and herbaceous plant species and has been used

mainly for those plant cell wall polysaccharides which are extractable by alkaline solutions (Margolles-Clark, 1996).

Hemicellulose consists of short, highly branched chains of sugars. It contains five-carbon sugars (usually D-xylose and L-arabinose) and six-carbon sugars (D-galactose, D-glucose and D-mannose) and uronic acid and the sugars are highly substituted with acetic acid. This branched nature renders hemicellulose amorphous and relatively easy to hydrolyse to its constituent sugars. When hydrolysed, the hemicellulose from hardwoods releases products high in xylose (a five-carbon sugar) whereas softwoods yield more six-carbon sugars (Kuhad *et al.*, 1997). Hemicelluloses are usually classified according to the main sugar residues in the backbone of the polymer e.g. as xylans (β-1,4-linked D-xylose units), and mannans (β-1,4-linked D-mannose units) which are the main groups and arabinans (α-1,5-linked L-arabinose units) and galactans (β-1,3-linked D-galactose units) which are less abundant (Margolles-Clark, 1996).

Xylans are present in all terrestrial plants and comprise up to 30% of the wall material of annual plants (grasses and cereals), 15 – 30% of hardwoods and 7 – 10% softwoods (Wilkie, 1979). Galactoglucomannan is the most abundant hemicellulose of softwoods where it can comprise up to 20% of the wood (Puls & Schuseil, 1993) whereas in hardwoods the content is only 2 – 5% of the cell walls (Tenkanen, 1995). Glucomannans and galactomannans also occur in annual plants, especially in seeds, tubers and bulbs, where they function as reserve polysaccharides (Aspinall, 1959).

1.7.4.2 Enzymatic degradation of hemicelluloses

The structure of the hemicellulose heteropolysaccharide is highly complex and a range of different enzymes are needed for their degradation and modification. The

depolymerisation of the hemicellulose backbone is accomplished by two main glycanases, *endo*-1,4-β-D-xylanase and *endo*-1,4-β-D-mannanase. Small oligosaccharides and substituents are split by various glucosidases such as β-D-xylosidase, β-D-mannosidase, α-L-arabinosidase, α-D-galactosidase and α-D-glucuronidase (Dekker, 1985). Esterases are needed in the total hydrolysis of xylans and mannans in addition to these glycosidic enzymes in order to remove acetic and phenolic acid side groups from sugar units (Biely *et al.*, 1985). In addition to these *endo*-enzymes which cleave bonds within the polymer, are *exo*-enzymes which normally cleave monomers or dimers from the end of the polymer. The products of hemicellulose degradation include carbon dioxide, water, cell biomass and a variety of small carbohydrate molecules including monomers and dimers.

Hemicellulosic compounds are subject to degradation by various fungal and bacterial populations (Atlas & Bartha, 1993). Microbial extracellular *endo*-1,4-β-xylanases have been widely studied and characterised in various filamentous fungi, bacteria and yeasts (Coughlan & Hazlewood, 1993; Dekker & Richards, 1976) whereas microbial *endo*-1,4-β-mannanases are mainly produced by fungi and bacteria (Dekker & Richards, 1976). Several microorganisms also produce multiple patterns of hemicellulose-degrading enzymes. The production of more than one enzyme with only slightly different catalytic properties is considered to be part of the strategy of microorganisms to maximise the utilisation of these heterogeneous substrates (Wong *et al.*, 1988). Although most studies to date have been undertaken with mesophilic microorganisms, this ability to produce enzymes with modified catalytic properties increases the likelihood that Antarctic microfungi will have adapted sufficiently to the demands of their environment to yield a variety of potentially novel or cold-active hemicellulases.

1.7.4.3 Applications of hemicellulases

The commercialisation of the hemicellulases centres largely on the xylanases and mannanases. These two enzyme groups have found an application in a broad range of industrial applications, the xylanases finding far more niches than mannanase. The earliest U.S. patent for a method of xylanase production was issued in 1979 for an enzyme mixture used as an animal feed additive for dairy cattle. Xylanase has since proven useful in many industrial applications (Table 1.5), many of which lend themselves to the use of a cold-acting enzyme.

Table 1.5 Industrial applications of xylanase and its advantages

Application	Advantages
Biobleaching paper pulp	Retains cellulose while removing lignin from paper pulp Achieves brighter results with chlorine-free bleaching (Kirk & Jeffries, 1996)
Animal feed	Stimulates growth rates by improving digestibility Improves quality of the animal litter (Beauchemin *et al.*, 1997)
Bread making	Makes bread fluffier with greater volume Improves crumb softness after storage
Juice making	Increases juice yield from fruits and vegetables by aiding maceration process Reduces viscosity and improves filterability
Beer making	Extracts more fermentable sugar from barley Processes spent barley for animal feed Reduces viscosity and improves filterability
Silage	Improves quality of silage Produces compounds that may be a nutritive source for ruminal microflora (Beauchemin *et al.*, 1997)
Organic waste disposal	Improves degradability of plant waste material
Detergents	Improves ability to remove fruit, vegetable and grass stains
Fuel-alcohol production	Decreases the viscosity of the mash Prevents fouling problems in distilling equipment
Vegetable oil extraction	Improves extraction of oil from oil-rich plant material
Linen manufacturing	Improves retting of flax fibres

As mentioned above, the industrial use of mannanase is not nearly as broad as that of xylanase, however it has established a niche, in many cases as part of an enzyme cocktail. In the pulp and paper industry, it has found application in association with xylanase (Buchert *et al.*, 1992). It is used in conjunction with pectinase and xylanase in the fruit

juice industry to reduce the viscosity of the juice and improve juice clarity. In addition, mannanase has found a considerable degree of acceptance as an additive to poultry feed where it contributes to improved egg weight and egg production.

A more recent and novel use of β-mannanase is its introduction by Procter & Gamble into their range of liquid laundry detergents (Anon., 2001). The mannanase detergent additive, Mannaway™, was developed by the major enzyme producer Novozymes and is claimed to be endowed with new capabilities, including the highly effective removal of stains left on fabrics by e.g. chocolate and ice-cream. The original organism expressing the mannanase enzyme was collected from soil samples 3,500 metres above sea level in northern Kashmir. At this elevation, the region would be well above the winter snowline where temperature and conditions would be similar to those found in coastal Antarctica. In developing their product for market, Novozymes transformed the mannanase gene from its original host (unidentified) into a *Bacillus* strain used in the production of a number of their enzymes.

1.7.5 Lipase

Lipolytic enzymes are ubiquitous in nature being widely distributed throughout all kingdoms of life (Fojan *et al.*, 2000), however, they are found more abundantly in microbial flora – bacteria, filamentous fungi and yeast (Jaeger *et al.*, 1994). Fungi produce different classes of these enzymes, in particular, carboxyl esterases (EC 3.1.1.1), which hydrolyse short chain carboxylic acids and lipases (triacylglycerol acylhydrolases, E. C. 3.1.1.3), which catalyse the hydrolysis and synthesis of long chain acylglycerides. The distinction between long and short chain fatty acids remains moot, some investigators ascribing the threshold for long chain as $C \geq 10$ (Sunna *et al.*, 2002) whilst others remain more conservative electing $C \geq 12$ (Eggert *et al.*, 2000). However, there is

unanimity in classifying lipases as enzymes which are capable of catalysing the hydrolysis of water insoluble (or poorly soluble) triacylglycerides to free fatty acids and glycerol at the water/oil boundary (Fig 1.8) (Brush *et al.*, 1999; Brzozowski *et al.*, 2000; Fojan *et al.*, 2000; Pandey *et al.*, 1999; Reetz, 2002; Schmidt-Dannert, 1999; Sharma *et al.*, 2001; Sunna *et al.*, 2002). The three dimensional structures of a number of lipases and esterases have been elucidated and all have been shown to be members of the α/β hydrolase superfamily (Fojan *et al.*, 2000; Jaeger *et al.*, 1999; Schmidt-Dannert, 1999; van Pouderoyen *et al.*, 2001). Lipases display little activity in aqueous solutions containing soluble substrates, whereas esterases show normal Michaelis-Menten kinetics in solution (Sharma *et al.*, 2001).

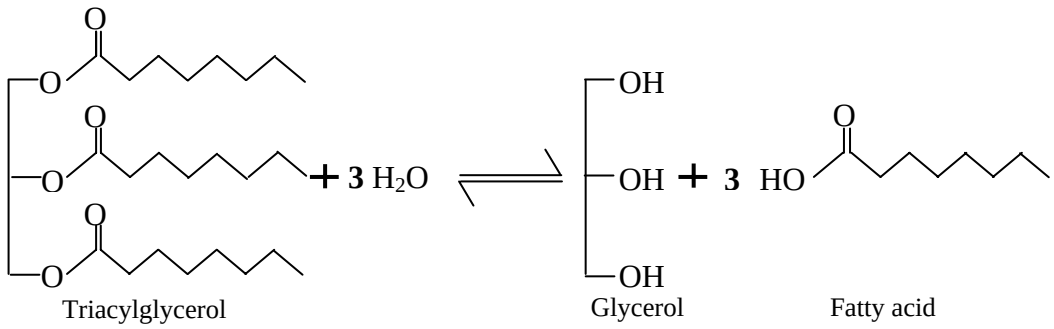

Figure 1.8 The catalytic action of lipases
A triglyceride can be hydrolysed to form glycerol and fatty acids, or the reverse (synthesis) reaction can combine glycerol and fatty acids to form a triglyceride (Jaeger & Reetz, 1998)

Lipases are enzymes of considerable physiological significance and industrial potential. In eukaryotes, lipases are involved in various stages of lipid metabolism including fat digestion, absorption, reconstitution and protein metabolism. In plants, lipases are found in energy reserve tissues. How lipases and lipids interact at the interface is still not clear and is a subject of intense investigation (Balashev *et al.*, 2001).

Lipases have found applications in numerous industrial areas. The most commercially important field of application for hydrolytic lipases is as additives in household and industrial laundry detergents and in household dishwasher detergents with sales in 1995 estimated to be valued at US$30 million (Jaeger & Reetz, 1998). Lipases also enjoy applications in the food industry – in the production of a variety of products, ranging from fruit juices, bakery foods and vegetable fermentation to the production of desirable flavours in cheese and other foods (Pandey *et al*., 1999; Sharma *et al.*, 2001). In the pulp and paper industry, lipases are used to remove 'pitch' from the pulp produced for paper making (Jaeger & Reetz, 1998). Additionally, lipases have applications in cosmetics and pharmaceutical processing (reviewed by Sharma *et al.*, 2001, and Pandey *et al*., 1999) and are the most widely used enzymes in synthetic organic chemistry, catalysing the chemo-, regio- and/or stereoselective hydrolysis of carboxylic acid esters or the reverse reaction, in organic solvents (Reetz, 2002). This use of lipases in fine chemical applications is largely due to the advantages of these catalysts for the production of optically pure compounds (Demirjian *et al.*, 2001).

Development of lipase-based technologies for the synthesis of novel compounds is rapidly expanding the uses of these enzymes (Liese *et al.*, 2000). A limiting factor, however, is the shortage of lipases having the required processing characteristics. Many new and novel lipases remain to be discovered and for this reason the prospecting for novel lipase genes is of interest from the industrial and academic standpoint. The extreme environmental conditions to which Antarctic organisms are subjected, calls out for an examination to be made of its microfungi in the search for new and different lipolytic enzymes, particularly for cold-adapted enzymes which have high potential value for the food, detergent and chemico-pharmaceutical industries.

1.8 Isolation of genes from microfungi

There is an ever-growing demand by industry for the identification and production of novel enzymes which can improve process performance or fulfil niche requirements in the production train. Many of these are hydrolases for which examples have been given above in Tables 1.2, 1.3 and 1.5. Genes encoding novel enzymes are widely prospected from microorganisms and filamentous fungi have proved a fruitful source (see Table 1.2)

1.8.1 Methods used for gene isolation

A number of techniques have been utilised to isolate a gene of interest from a microfungus or other organism. A gene library can be constructed by the partial digestion of gDNA with a restriction enzyme, ligated with a suitable phage or plasmid vector and introduced into a bacterial host, usually a strain of *Escherichia coli* (Sambrook *et al.*, 1989). Another method involves the construction of a cDNA library. Total RNA isolated from a target organism producing the gene product of interest can be used as a template to synthesize double-stranded cDNA with reverse transcriptase PCR. Additionally, with the aid of degenerate primers based on conserved regions of purified target proteins, PCR techniques have been used to amplify whole genes of interest (Bell *et al.*, 2002; Ding & Buswell, 2001; Ozturk & Ogel, 2000). Once again, a library can be constructed from the PCR products by ligating and cloning into *E. coli* as previously or the entire gene isolated by genomic walking PCR (Morris *et al.*, 1995; 1998). As an alternative to *E. coli*, S*accharomyces cerevisiae* has also been used for the expression host for cDNA libraries (Dalboge, 1997; Saloheimo *et al.*, 1993; 1997). Bacterial artificial chromosome (BAC) libraries can be constructed from large fragments of DNA (40 – 200 kbp and greater) isolated from environmental samples (Rondon *et al.*, 2000) or from a particular organism (Lijavetzky *et al.*, 1999), ligated into plasmid vectors and cloned into *E. coli* (Shizuya *et*

al., 1992). Once constructed, the BAC libraries can be prospected for gene products of interest (reviewed in DeLong, 2002).

The libraries created need to be screened for expression of the protein of interest. The method of recognising the presence of a sought after protein must naturally be dictated by that protein's unique characteristics. In many cases the individual recombinant bacterial or yeast clones can be screened directly using indicators in the growth medium (such as discussed in 1.7.3). Filamentous fungal genes encoding for cellulases have been isolated by differential hybridization (Penttilä *et al.*, 1986; Saloheimo *et al.*, 1988; Shoemaker *et al.*, 1983; Teeri *et al.*, 1983) and genes coding for lignocellulose hydrolysing enzymes by using heterologous genes as hybridisation probes (Ahn *et al.*, 2001; reviewed in Nevalainen & Penttilä, 1995; Takashima *et al.*, 1996).

1.8.1.1 Special considerations when isolating lipase genes from fungi

The active site of α/β hydrolases contains a catalytic triad consisting of conserved serine, aspartic/glutamic acid and histidine residues (Bornscheuer *et al.*, 2002; Jaeger *et al.*, 1999). Despite sharing a common catalytic mechanism and structure, lipases share little similarity with each other at the amino acid level (Brzozowski *et al.*, 2000). Although the low homology observed between different lipase genes makes them difficult targets for PCR, Bell *et al*. (2002) have developed an alternative method of identifying and isolating novel lipases with PCR by amplify small regions of the gene directly from bacterial gDNA using specially designed primers. By designing highly degenerate consensus primers to the oxyanion hole (Jaeger *et al.*, 1994) and the active-site regions, it was found possible to amplify fragments of putative lipase genes. Using genomic-walking PCR (Morris *et al.*, 1995; 1998) the complete lipase gene sequence could be obtained from a mixed environmental DNA sample.

Once a fungal lipase gene has been wholly identified, specific primers can be designed to permit the whole gene to be amplified by PCR and cloned into a suitable expression vector by conventional means.

1.8.2 Expression of novel genes in heterologous hosts

When a novel protein is identified, it invariably originates from a host which is not recognised as an industrially exploited organism or strain. Any developments with this native host carries with it the potential complications and expense of complying with the various health and safety regulations imposed by legislative authorities. Even setting aside such legislative restrictions, the level of output of the targeted protein by its native host is generally too low to make its exploitation economic.

In order to overcome these problems, the gene encoding a novel protein can be transformed into a recognised production organism. There are a presently a range of bacterial and fungal organisms utilised as heterologous production hosts. However, if endeavouring to exploit a fungal gene, the most appropriate expression host is another filamentous fungus such as *Aspergillus* sp. or *Trichoderma* sp.

1.8.2.1 Selection of a promoter

In order to obtain the maximum yield of the gene product from the heterologous host, it is essential that the transcription of the DNA be under the control of a strong promoter. Gene promoters used for recombinant expression can be either inducible or constitutive. As most of the strong production promoters are derived from genes encoding extracellular hydrolytic enzymes such as cellulases and amylolytic enzymes, protein production in filamentous fungi is controlled fairly strongly by transcriptional regulation.

Constitutive production of these enzymes would be a waste of energy for the fungus. Among the strongest inducible promoters are the glucoamylase A promoter (*glaA*) of *A. niger* var. *awamori* (Ward et al., 1990) and the *T. reesei* cellobiohydrolase I (*cbh1*) promoter (Harkki *et al.*, 1991; reviewed in Paloheimo *et al.*, 1993; Penttilä, 1998). Production using the *cbh1* promoter can be obtained on media containing cellulose, plant material or lactose (Kubicek & Penttilä, 1998). *T. reesei* has been used successfully as a host for heterologous fungal proteins (de Faria *et al.*, 2002; reviewed in Keränen & Penttilä, 1995; Penttilä, 1998; Saarelainen *et al.*, 1997) with the heterologous genes being expressed using the *cbh1* promoter.

1.8.2.2 Transformation strategies

A number of genetic transformation systems have been developed for filamentous fungi (Finkelstein, 1992; Irie *et al.*, 2001 and references therein). A traditional method successfully applied for a large number of filamentous fungi is polyethylene glycol mediated DNA uptake of protoplasts (reviewed in Gokhale, 1992). Other previously used methods include electroporation of protoplasts (Goldman *et al.*, 1990; Ward *et al.*, 1989) and incubation of germinating conidia in a lithium salt (Dhawale & Marzluf, 1984). However, the more recently introduced method for the integration of a gene in a (heterologous) fungal host is microprojectile bombardment of intact conidia using gold or tungsten particles coated with the transforming DNA (Armaleo *et al.*, 1990; Hazell *et al.*, 2000; Herzog *et al.*, 1996; Lorito *et al.*, 1993; Te'o *et al.*, 2002). The biolistic method successfully bypasses the time consuming step required for the purification of transformed protoplasts and yields a relatively high percentage of stable transformants.

A selection marker can be introduced into the fungal host by including it in the same vector as the gene or alternatively by introducing it by cotransformation. A number of

dominant selection markers are available, for example, the bacterial antibiotic resistance markers, such as the *hph* gene encoding resistance to hygromycin B (Punt *et al.*, 1987) or the *A. nidulans amdS* gene (Kelly & Hynes, 1985) encoding the acetamidase enzyme. Integration of the gene into the genome of the host can be ascertained by PCR using suitable primer sequences. To determine if the gene is functional in its new host, activity can be assayed by culturing transformants on selective solid media as described in 1.7.3 or in a liquid medium designed to induce production of the recombinant under the control of the chosen promoter.

1.9 Aims of this study

The present study consists of a number of steps each directed towards the common goal of evaluating Antarctic microfungi as a potential bioresource. An overview of the strategy is provided in Fig 1.9.

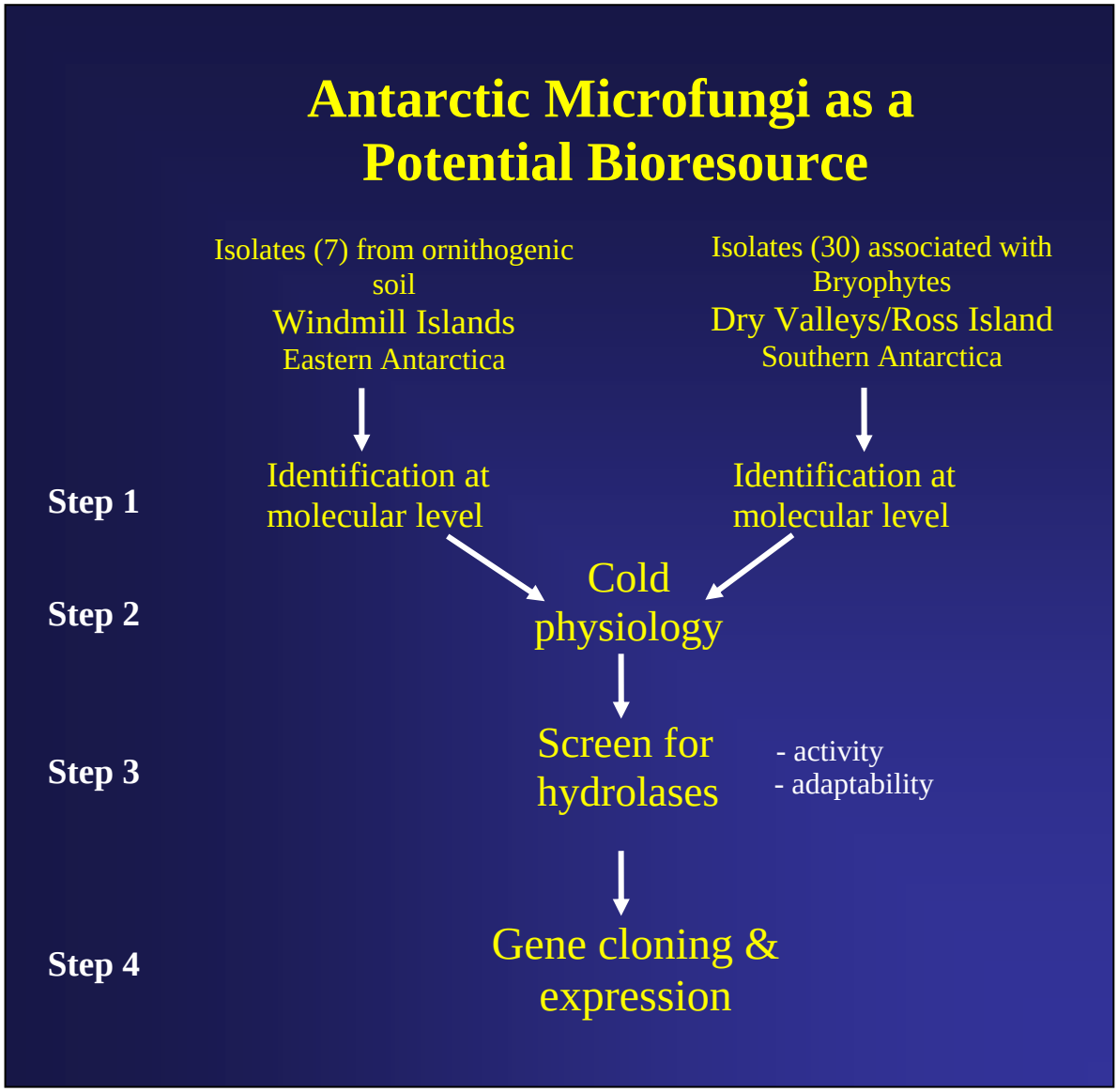

Figure 1.9 **An overview of the strategy applied in this study**

- The first step involved the identification of the microfungal isolates obtained from two separated regions on the Antarctic continent and was aimed at establishing the potential of Antarctica as a source of varied microfungal species.

- The second step embraced an examination of some physiological aspects of Antarctic filamentous microfungi and was directed at identifying features which may contribute significantly to cold survival.

- The third step involved screening the fungal isolates to determine their potential as a source of cold-adapted hydrolytic enzymes and to characterise two hemicellulases, β-mannanase and xylanase, secreted by a sub-set of the microfungi.

- The fourth and final step evaluated the potential for isolating novel genes from Antarctic microfungi and for their cloning and expression in an industrially exploited mesophilic heterologous host.

2. Materials and Methods

Detailed information is provided in this section for new subject matter and for materials or methods not included in original publications (Appendices I – V). Where information is available in an original publication, the appropriate Appendix will be referenced.

2.1 Fungal strains and cultivation conditions

Antarctic fungal strains were obtained from two locations on the Antarctica continent (see Figs. 1.1, 2.1 & 2.2), from the Windmill Islands (lat 66°17'S, long 110°32'E) situated offshore near the Australian Antarctic Research Station at Casey in Wilkes Land and from four sites around Ross Island and McMurdo Sound in Victoria Land (approx lat 77°30'S, long 170ºE). All strains were stored on potato dextrose agar (PDA) slants held at 4^{o}C.

2.1.1 Ornithogenic species

Seven isolates were provided by Dr. R. D. Seppelt of the Australian Antarctic Division, from a collection obtained from the Windmill Islands during the austral summers of 1994/95 and 1995/96 (Fig 2.1). Theses isolates were collected from ornithogenic soils in penguin colony areas and were tentatively identified by Dr. Seppelt based on their morphology. The seven strains consisted of three *Penicillia*, nominated as *Penicillium* lake isolate, *Penicillium* sp2 and *Penicillium* sp17, *Alternaria alternata*, *Phoma* sp. and two *Trichoderma* isolates sp10 and sp10-b.

2.1.2 Bryophytic species

The remaining Antarctic isolates were recovered from moss (bryophyte) samples (see Fig 1.2) collected by Dr. P. M. Selkirk (Macquarie University) and Dr. M. L. Skotnicki (Australian National University) from sites on Ross Island and Dry Valleys region in

January 1995 and December 1996 (Table 2.2). Moss samples were dried and stored at ambient temperature in Antarctica (approximately 0ºC) until transport to Australia, then stored at –18ºC prior to examination. Microfungi were isolated from the moss samples as described in Appendix III.

Table 2.1 Bryophytic sources of microfungal isolates

Bryophyte species	Date collected	Description of collection site	Microfungal isolates*
Bryum argenteum	17 Dec 96	Marble Point. Gently flowing water in Surko Stream	Refer App III
Hennediella heimii (moribund)	12 Dec 96	Cape Chocolate. North of stream (CC16)	1b, 1d
H. heimii	12 Dec 96	Cape Chocolate. Open valley (CC14)	nil
B. argenteum	19 Dec 96	Marble Point. Dry turf near Surko Stream (CP1)	3a-2, 3R
B. argenteum	19 Dec 96	Marble Point. Between Surko Stream and air facility (CC52)	4a, 4b-1
H. heimii	19 Dec 96	Marble Point. Near air facility (CC54)	5R, 5G, 5a, 5a-1, 5a-3, 5b, 5g
H. heimii	19 Dec 96	Marble Point. Between Surko Stream and air facility (CC52)	6G, 6a-1, 6a-2
B. argenteum	13 Jan 95	Cape Royds North (T557)	7G, 7a-1, 7b-1
H. heimii	12 Dec 96	Cape Chocolate (C118)	8R
B. argenteum	11 Jan 95	Cape Crozier (T489)	9a, 9a-R, 9b, 9b-R
H. heimii	17 Dec 96	Marble Point. North side of rocks (C239)	10R, 10G, 10a-1

*The microfungal colonies recovered were primarily identified with the alpha numeric code shown here, based on the bryophyte sample number (arbitrarily numbered 1 - 10) and a plate code. The isolate ID is a concatenation of the collection site code and the colony ID (see Table 3.2).

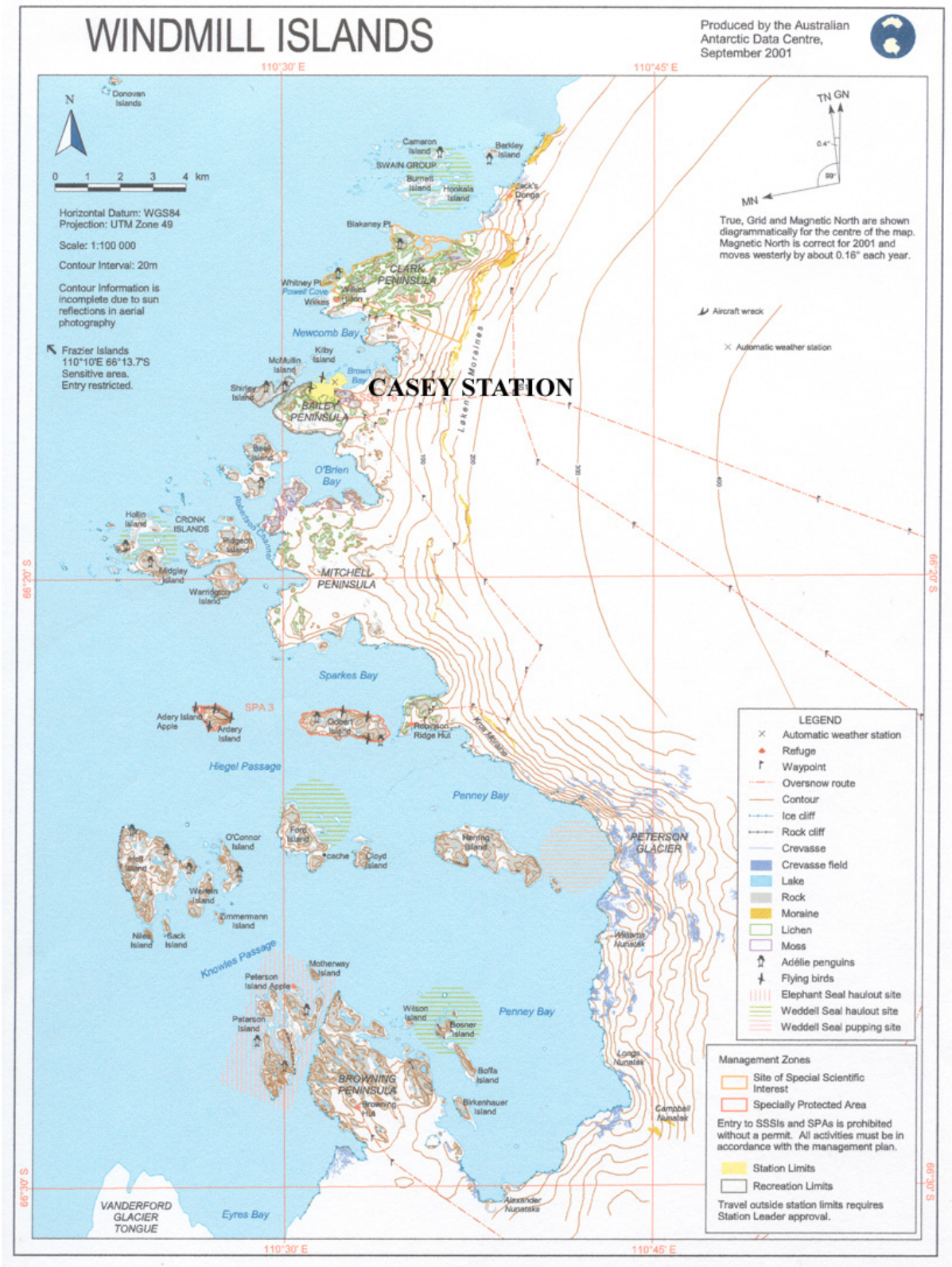

Figure 2.1 **Windmill Islands, Wilkes Land, Antarctica. Source of microfungi associated with ornithogenic soils**

Figure 2.2 **McMurdo Sound and Ross Island, Victoria Land, Antarctica. Sources of microfungi associated with bryophytes (indicated by red boxes)**
From Joan Myers *Antarctic Journal* http://www.joanmyers.com/Jnlinset.htm

2.1.3 Mesophilic species

Microfungal material used for control and comparative purposes are listed in Table 2.2.

Table 2.2 Mesophilic microfungal isolates used in this study

Fungi	Strain ID	Reference	Fungi	Strain ID	Reference
Penicillium restrictum	DAR38028	3.1.2	*Trichoderma reesei*	VTT-D-79125	App II & App V
Penicillium decumbens	DAR38025	3.1.2.	*Trichoderma viride*	ALKO3092	App IV & 3.3.1
Alternaria alternata	DAR45878	3.1.2.	*Penicillium expansum*	DAR27044	App IV
Phoma herbarum	DAR57083	App IV, 3.1.2 & 3.3.1	*Penicilium janthinellum*	DAR45887	3.1.2
Trichoderma viride	DAR28047	3.1.2	*Candida albicans*	MU	3.1.2
Trichoderma hazianum	DAR41916	3.1.2	*Embellisia* sp.	DAR58941	App III & 3.1.2
Epicoccum nigram	MU	3.1.2	*Curvularia brachyspora*	DAR68403b	App III
Aspergillus terreus	DAR41919	3.1.2	*Curvularia trifolii*	DAR24953	App III
Rhizopus stolonifer	DAR27904	3.1.2			

References 3.1.2 and 3.3.1 refer to chapter 3 in this work
DAR – NSW Agriculture, Plant Pathology Herbarium, Orange, NSW, Australia
VTT – State Technical Research Centre, Espoo, Finland
ALKO – Roal Oy, Rajamäki, Finland
MU – Macquarie University

2.2 Molecular identification of fungal isolates

2.2.1 DNA extraction

Genomic DNA (gDNA) was isolated directly from the mycelial mass of fungal isolates grown on PDA plates at 28ºC for 5-7 days using the FastPrep ™ FP120 bead beating machine (Bio101, CA, USA) in accordance with the manufacturers directions (refer Apps. III, V).

2.2.2 SSU nrDNA (18S) gene

PCR Amplification – The fungal SSU nrDNA (18S) gene was amplified using the primers, 18S forward (5´-AACCTGGTTGATCCTGCCAG) and 18S reverse (5´-CCTTCTGCAGGTTCACCTAC), which lie immediately inside the 5´ and 3´ ends

respectively of the 18S gene. For the PCR, 1 µl of DNA (from 2.2.1) was mixed with 9 µl of Genereleaser™ (Bioventures Inc., Murfreesboro, TN, USA) in a 0.5 ml tube and overlaid with two drops of sterile mineral oil. DNA samples with Genereleaser™ were heated in a microwave oven as previously described (Gillings & Holley, 1997). Negative controls containing water only and Genereleaser™ only, were included with each set of reactions. Tubes were incubated at 80ºC before PCR mastermix (40 µl) was added. Final concentrations of reagents were as follows: 20 mM $(NH_4)_2SO_4$, 75 mM Tris-HCl (pH 9.0), 0.1% (w/v) Tween-20, 2 mM $MgCL_2$, 0.2 mM of each primer, 0.2 mM of each deoxyribonucleotide triphosphate plus 1.5 U Red Hot DNA Polymerase (Advanced Biotechnologies, Surrey, UK). In addition, each reaction tube contained 20 ng RNAse A to degrade any RNA that may be present. PCR was performed in an Omn-E PCR Thermal Cycler (Hybaid Limited, UK) under the following conditions: [1x] 94ºC, 3 min (premelt); [35x] 94ºC, 30 s (denature); 58ºC, 30 s (anneal); 72ºC, 1 min 30 s (extension); [1x] 72ºC, 5 min (final extension).

DNA Analaysis (RFLP) – Results from the PCR amplification were analysed from 10 µl aliquots by electrophoresis on a 2% (w/v) agarose gel in TBE buffer (pH 8.3) (Sambrook *et al*. 1989). Each of the PCR amplicons was subjected to restriction enzyme digestion separately by *Hin*f I and *Msp* I (Progen [MBI Fermentas], Qld, Australia). The gels were stained with ethidium bromide, photographed using transmitted uv light and Polaroid film (Sambrook *et al*. 1989) and the digestion pattern was analysed. A 100 base pair ladder (Pharmacia, LKB, Milwaukee, USA) or kilobase ladder (Promega Corporation, Madison, WI, USA) was included on every gel.

DNA Sequencing – The 18S PCR product (1 µl) was ligated into a pGEM®-T Vector (Promega) and transformed into competent *Escherichia coli* JM109 cells in accordance with the manufacturer's instructions.

To confirm the inclusion of the 18S fragment into successful transformants (white colonies on LB/ampicillin/IPTG/X-gal plates), a sterile toothpick was used to pick cells from the colony and these were introduced to 100 µl of sterile water in a 1.5 ml micro centrifuge tube. After vortexing, 5 µl of the solution was removed to a sterile 0.5 ml tube and two drops of mineral oil added. The tube was heated to 98ºC for 10 min and held at 80ºC until 40 µl of master mix (as described above) could be added and PCR commenced following the same thermal protocol described above. Successful inclusion of the fungal 18S fragment into the transformant was indicated by the presence of a 2 kbp band (approximately) when the amplification product (10 µl) was run on a 2% agarose gel.

McCartney bottles containing 3 ml L-broth were inoculated from the successful transformants and incubated overnight at 37ºC on a rotary shaker (150 rpm). Plasmid DNA was purified from the bacterial culture using the Wizard™ *Plus* Minipreps DNA Purification System (Promega) in accordance with the manufacturer's instructions. Successful recovery of plasmid DNA was confirmed by electrophoresis of the cleaned product (3 µl) on a 0.8% agarose gel.

Five primers were used to sequence the 18S nrRNA which was performed on an ABI Prism, Model 373A automated fluorescent DNA sequencer (Perkin-Elmer). Sequencing, commenced from 5'– and 3'– ends using the primers pGEM-F and pGEM-R (Promega) respectively. To complete the sequencing of the 18S nrDNA, three internal sequencing primers were designed: forward primer 18SF390 (5´-GGAGCCTGAGAAACGGCTAC)

and reverse primers 18SR360 (5´-ATCACAGACCTGTTATTGCC) and 18SR800 (5´-CGGCATAGTTTATGGTTAAGA).

Sequence Analysis - Comparison and alignment of 18S DNA sequence data was performed using the ANGIS - Bioinformatic Service suite of programs (http://morgan.angis.su.oz.au/new/bioinformatic/index.html).

2.2.3 Internal transcribed spacer (ITS) region on nrDNA

PCR Amplification – The ITS region of the nrDNA gene was amplified with PCR conditions (using AmplTaq Gold polymerase; Applied Biosystems) as follows: [1x] 94ºC, 12 min; [35x] 94ºC, 1 min; 58ºC, 1 min; 72ºC, 1 min; [1x] 72ºC, 5 min (refer App III) utilising the primers ITS1 (5´- TCCGTAGGTGAACCTGCGG) and ITS4 (5´-TCCTCCGCTTATTGATATGC) (White *et al.*, 1990). When using Eppendorf Taq DNA polymerase, the 12 min premelt and activation step was reduced to 2 min (in accordance with each of the manufacturers' instructions). As an alternative, the forward primer ITS5 (White *et al.*, 1990) (5´-GGAAGTAAAAGTCGTAACAAGG), located slightly upstream of ITS1 in the 18S conserved region, was used in place of ITS1. PCR products were cleaned and sequenced as described in Appendix III.

Sequence Analysis – Comparison and alignment of ITS sequence data was again performed using the ANGIS suite of programs. Phylogenic analysis of aligned sequences was performed using the PHYLIP (Phylogeny Inference Package) computer programs, version 3.57 (refer App III).

2.3 Fungal physiology

The isolates listed in Table 2.3 were assayed to identify the levels of activity involved in the production of compounds implicated in physiological processes.

Table 2.3 Fungal isolates assayed for physiological activity

Isolate	Origin
Penicillium expansum DAR74612	Windmill Islands, Antarctica
Penicillium expansum DAR27044	Bathurst, NSW (mesophilic)
Trichoderma viride DAR74618	Windmill Islands, Antarctica
Trichoderma viride ALKO3092	VTT, Finland (mesophilic)
Phoma sp2 DAR74616	Windmill Islands Antarctica
Phoma herbarum DAR57083	Chiswick, NSW (mesophilic)
Embellisia sp2 DAR74619	Marble Point, Antarctica
Embellisia sp. DAR58941	Albion Park Rail, NSW (mesophilic)

2.3.1 Trehalose production

Preparation of assay material – Mycelial samples (about 1 cm^2) were removed from PDA store plates for each of the fungal isolates and transferred to cellophane discs covering the surface of PDA plates. Samples were grown at room temperature (23-24ºC) until well established (8-10 days) prior to harvesting. For samples to be assayed following cold treatment, plates were transferred to a refrigerator (4-5ºC) where incubation was continued for a further 10 days.

Approximately 300 mg of fungal mycelia were removed from the cellophane discs and transferred to a 2 ml screw cap tube containing glass beads of various grades (Bio101). One ml of 80% ethanol was added to each tube and after boiling for 30 min in a boiling water bath, tubes were placed in a FastPrep™ FP120 bead beating machine (BIO101) and agitated (2x) at 5 m s^{-1} for 30 s. Tubes were centrifuged at 13,000 rpm for 5 min in a

microfuge and 2 x 400 µl of cell free supernatant was removed to 0.6 ml tubes. The supernatant was totally evaporated by heating the tubes at 60ºC for 24 h. The resultant pellets were combined, resuspended in 400 µl of sterile water and stored at -20ºC until assayed.

Protein content of the samples was determined using the Bio-Rad DC Protein Assay Kit (Bio-Rad Laboratories).

Determination of trehalose content – The trehalose was digested in a 200 µl reaction volume containing 50 µl of sample and 10 µl of trehalase in the presence of 50 mM sodium acetate buffer (pH 4.5) at 50ºC for 30 min. The reaction was stopped by transferring the samples to a water bath and boiling for 5 min (Hazell, 1999). Samples containing water instead of trehalase were also assayed to ensure that residual glucose in the sample was accounted for. The resultant glucose was determined using the Boehringer Mannheim D-glucose Detection Kit (Cat No 139 106) in accordance with the manufacturer's instructions but with a scaled down final volume of 302 µl.

2.3.2 Glycerol production

Preparation of assay material – Material prepared for trehalose assays (2.4.1) was also utilised for the determination of glycerol production.

Glycerol determination – The assay for glycerol was performed by reacting 100 µl of sample in a volume of 1 ml which contained 50 mM Tris (pH 8), 2.5 mM phosphenoenolpyruvate (Boehringer Mannheim, Cat No 128 082), 1.25 mM ATP (Boehringer Mannheim, Cat No 127 523), 1.25 mM $MgSO_4$, 300 µM NADH (Boehringer Mannheim, Cat No 128 023), 5 U lactate dehydrogenase (Boehringer Mannheim, 127 230), 4 U pyruvate kinase (Boehringer Mannheim, Cat No 128 155) and 1 U

glycerolkinase (Boehringer Mannheim, Cat No 691 836). The samples were vortexed then incubated for 15 min at 30ºC. The difference in absorbance at 340 nm (A_{340}) between the samples and a control lacking glycerokinase was measured by a spectrophotometer (Hazell, 1999).

2.3.3 Antioxidant activity

Qualitative assessment – Spores from each of the isolates (except *Embellisia* sp. for which mycelium was used) were introduced to 50 ml of PD broth in 250 ml shake flasks and incubated on a rotary shaker at room temperature (23-24ºC) and 250 rpm until mycelia were well developed (4 days). A sample of growth medium containing mycelia was transferred from each flask to a 1.5 ml Eppendorf tube and refrigerated to -20ºC until frozen.

The frozen tubes were removed from the freezer and thawed (under refrigeration at 4-5ºC). A second 500 µl sample was removed from the shake culture and both samples were stained with 1 µl of stock dihydroethidium (refer App IV). A small section of stained mycelia was removed from each sample and placed on a microscope slide which was examined by fluorescence microscopy using a blue monochromatic light source (488 nm) and photographed.

Quantitative assessment – In an attempt to quantify any change in the level of oxidative activity present in the microfungal mycelia when cultured under stress conditions, spores from isolates were cultured in liquid medium (PD broth) at both room temperature and at 5ºC. As no spores could be obtained from the mesophilic *Embellisia* sp., this isolate and its Antarctic equivalent were omitted from this experiment.

Samples (500 µl) were removed from each of the culture shake flasks at the commencement of the experiment and hourly once germination was observed to have commenced (light microscopy). Each of the samples was stained with dihydroethidium (as above) and processed through a Becton Dickinson FACSCalibur flow cytometer (see App IV). The cultivation temperature of each sample was maintained until processed.

2.3.4 Flow Cytometry

Details of the development and application of flow cytometry to the analysis of fluorescently stained microfungi are provided in Appendix IV.

2.4 Hydrolase activity of secreted proteins

2.4.1 Screening for hydrolase activity on solid media

Appendix I provides a brief description of the materials and methods required for the screening for hydrolase activity. The following section expands on this material.

Preparation of plates containing the growth substrate – All isolates were screened on minimal medium plates (MM) containing 1.5% (w/v) KH_2PO_4, 0.5% (w/v) $(NH_4)_2SO_4$, 0.06% (w/v) $MgSO_4 \cdot 7H_20$, 0.06% (w/v) $CaCl_2 \cdot 2H_2O$ and minimum nutrient salts (Mandels & Weber, 1969; Nevalainen, 1981). In all plates, 2% (w/v) agar (Difco Bacto Agar) was included as solidifying agent and pH was adjusted to 5.5 with 1 M KOH. To keep the colony sizes reasonably small over the incubation period of several days without otherwise interfering with their morphology, 0.01 % (v/v) Triton X-100 was added into the medium (Nevalainen, 1981). The substrates listed in Table 2.4, appropriate to the hydrolytic activity being screened, were included in the plating medium as described in the references. Birch xylan and AZCL-pachyman were mixed with the plating medium

prior to autoclaving. Hydroxyethylcellulose was added to plates for some isolates as an alternative to Walseth cellulose.

Screening procedure – Protease and cellulase (Walseth) activity were observed by the presence of a clearing zone (halo) around the fungal colony, indicating the secretion of the enzyme into the growth medium and consequential hydrolysis of the substrate. For

Table 2.4 Substrates added into growth plates to indicate production of a particular hydrolase activity

All percentages are w/v with the exception of olive oil which is v/v.

Enzyme activity	Carbon source	Reference
Protease	2% glucose, 1% skim milk	Mäntylä *et al*. 1994
β-mannanase	0.5% locust bean gum	Rättö & Poutanen 1988
Xylanase	0.5% birch xylan	This work
lipase	2.5% olive oil, 0.001% rhodamine B	Kouker & Jaeger 1987
Chitinase	0.3% chitin	O'Brien & Colwell 1987
Pectinase	0.1% pectin	Hagerman *et al*. 1985
Cellulase	0.5% Walseth cellulose	Walseth 1952
	0.5% hydroxyethylcellulose	Nevalainen 1985
β-glucanase	0.1% AZCL-pachyman	This work

visualization of the xylanase, β-mannanase and cellulase (hydroxyethyl) activity, the screening plates were flooded with Congo Red (Sigma, 1 mg ml^{-1} in distilled water) for 5 min at room temperature and destained with 1 M NaCl for 15 min (Teather & Wood, 1982). A light yellow/brown halo around colonies on the deep red background indicated enzyme production. The β-glucanase activity was indicated by the diffusion of the AZCL dyed pachyman particles (Megazyme) and clearing zones around the colonies on the deep blue background. Chitinase activity from colonies growing on 0.3% (w/v) chitin was identified by a modification of the method of O'Brien & Colwell (1987). Three

milligrams of 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide (4-MUF-GlcNAc, Sigma) was dissolved in 0.6 ml of dimethylformamide and diluted with 9.4 ml of 0.1 M phosphate buffer. The solution was then added to the plates dropwise to cover the fungal colonies. Plates were incubated at 28^{o}C for 10 min and viewed under uv light. Chitinase (glucosaminidase) activity was indicated by the presence of a strong light-blue fluorescence around the fungal colony. Lipase activity was indicated by an orange fluorescent halo under uv light surrounding the fungal colony on plates containing rhodamine B and olive oil (Kouker & Jaeger, 1987). Pectin-containing plates were flooded with fresh 0.02% (w/v) ruthenium red (Sigma) in distilled water, refrigerated for 2 h and then destained for 15 min in water (Hagerman *et al.*, 1985). The presence of pectin lyase was indicated by the appearance of a clearing halo in a uniformly red stained background. Pectin methyl esterase activity was indicated by regions of a deeper red colour.

The general ability of a fungus to use a particular carbon source for growth under each temperature regime was determined by measurement of the diameter of the fungal colony on a screening plate. Similarly, the extent of enzyme activity secreted by each colony was obtained by measuring the clearing/fluorescing zone around the growing colony. The diameter of each colony and its activity zone were measured in two dimensions at 90º to each other and the values obtained averaged to give effective diameters. For each of the four replicates, diameters thus calculated were in turn averaged to yield the overall colony/activity diameter. Individual colony/activity diameters differed from the overall average by no more than 5%. These measures were then used to determine an index of relative enzyme activity (RA) for each fungal strain, substrate and temperature combination by dividing the total area of activity (the area of the clearing zone less the area of the colony) by area of the colony (Fig 2.3).

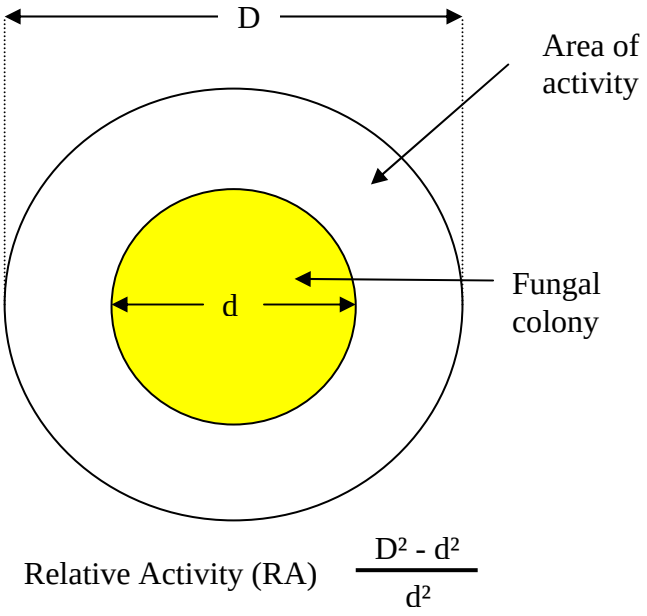

Figure 2.3 Determination of the relative enzyme activity index (RA)

2.4.2 Hemicellulase activity assays

Appendix II provides the materials and methods required for the assay of xylanase and mannanase activity in microfungal supernatant.

2.5 Gene cloning and expression

The materials and methods required to probe the genome of the Antarctic isolate *Penicillium allii* for a gene of interest (a lipase), isolate the target gene sequence and use biolistic bombardment to introduce the gene into a heterologous host, *Trichoderma reesei*, are provided in Appendix V.

2.5.1 Further characterisation of the lipase

In addition to characterising the secreted enzyme at room temperature, further assays of lipase activity were performed at 10ºC, 37ºC and 50ºC over a pH range from 3 to 10. Assays were performed using *p*-nitrophenyl caprate (C_{10}) as the substrate under the same

conditions as shown in Appendix V and also with *p*-nitrophenyl laurate (C_{12}) but with extended reaction time.

SDS-PAGE (Laemmli, 1970) was used to analyse supernatant from lipase cultures. Lipase activity was identified in gels which were renatured by incubating in 2.5% (v/v) Triton X-100 for 40 min (2x) then washed (3x) for 20 min in Milli-Q water. The renatured gels were stained overnight at room temperature with a solution consisting of 40 ml Universal buffer (120 mM, pH 6; Britton & Robinson, 1931) containing 20 mg Fast Blue BB Salt (Sigma) to which was added 200 µl of α-naphthyl acetate (Sigma) (20 mg ml^{-1} in acetone). The reaction was stopped with 7.5% (v/v) acetic acid then washed thoroughly with water (Higerd & Spizizen, 1973). Following incubation, gels were photographed and then restained with Coomassie Blue R250.

2.5.2 Protein modelling

Three dimensional protein modelling was performed using PDB files generated by ExPASy (**Ex**pert **P**rotein **A**nalysis **Sy**stem) (http://www.expasy.org/swissmod/) and analysed using DeepView - The Swiss-PdbViewer v.3.7 (http://www.expasy.org/spdbv/). Ramachandran plots were generated using RamPage (http://www-cryst.bioc.cam.ac.uk/rampage).

3. Results and Discussion

3.1 Microfungal identification

The microfungi obtained from the ornithogenic soils of the Windmill Islands had been tentatively identified prior to receipt (see 2.1.1). Fungal isolates were recovered from the bryophytes in two stages. An initial sample obtained from Marble Point was investigated to determine its potential as a source of microfungi and this yielded three isolates (App III). Based on this successful recovery of viable fungal species, the remaining ten bryophyte samples were examined and yielded a further 27 isolates (see Tables 2.1 & 3.2). A representative sample of the microfungi recovered from the bryophytes is shown in Fig 3.1. All photographs were taken of the fungi growing at 10ºC on PDA plates started from a small plug of material introduced to their centres, except for isolate 5b which was streaked onto the plate. The blue/green background colour that appears is an artefact of the digital photographs and was not present in the plate medium, however the purple pigmentation in isolate 5b was visible.

As a prelude to an investigation into the potential of Antarctic microfungi as a bioresource, it was appropriate to obtain some level of identification of the microorganisms under review.

3.1.1 Morphological identification of microfungi isolated from Windmill Islands and Marble Point

The initial classification of the seven isolates obtained from the Windmill Islands was based on their overall morphology, in particular the characteristic structure of their conidia or in the case of the *Phoma*, the presence of pycnidia. When the first bryophyte obtained from Marble Point was examined, two of the three isolates obtained were

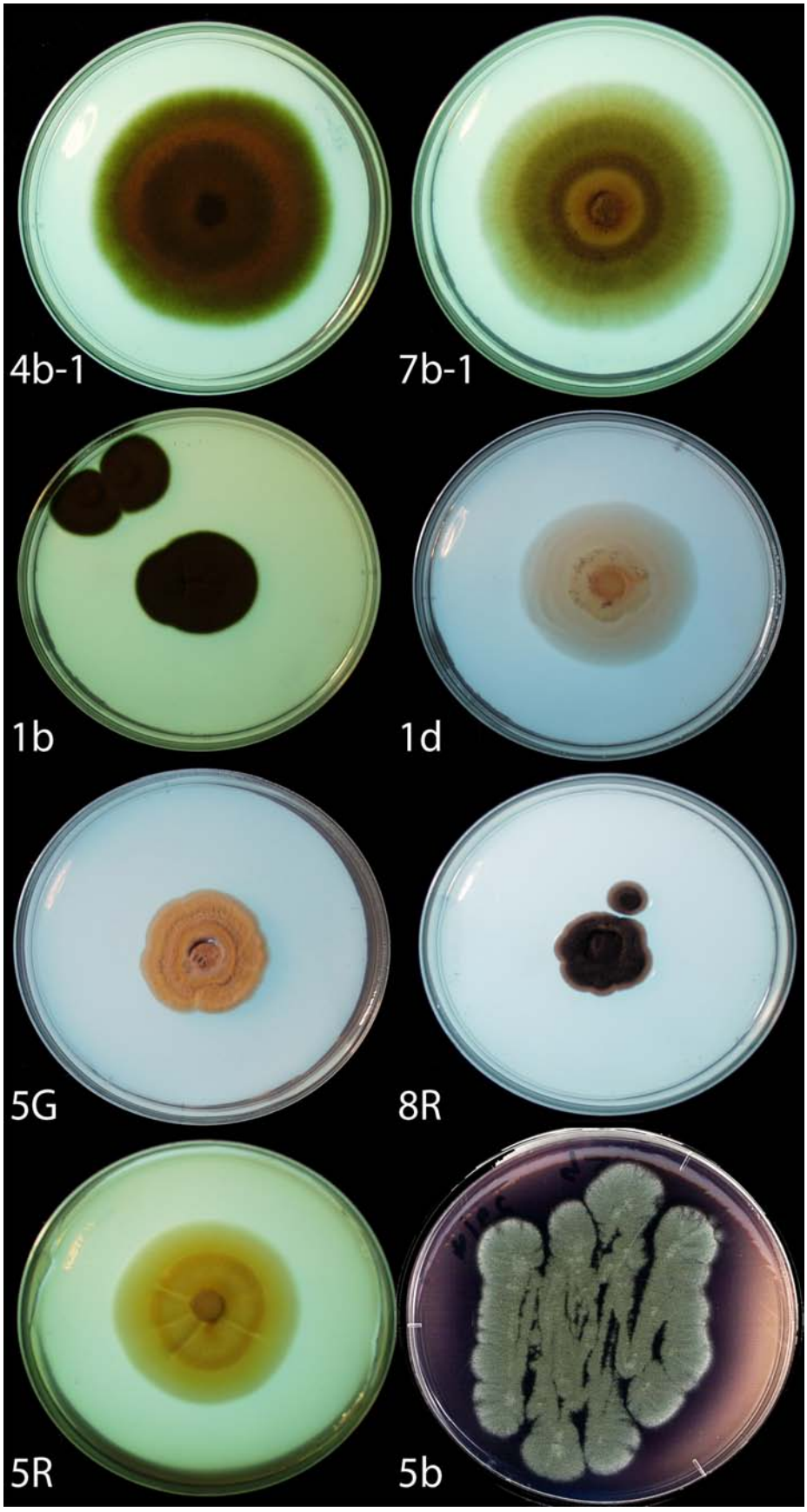

Figure 3.1 Digital photographs of microfungi recovered from Antarctic bryophytes

The label shown by each image refers to the source of the isolate (Table 2.1)

readily identifiable by their colony phenotype and conidiophore morphology as a *Penicillium* sp. and a *Trichoderma* sp. (App III). No further investigation was made of these two isolates. The remaining bryophyte isolate together with the three ornithogenic *Penicillia* were examined by reputable taxonomists, Dr A. D. Hocking, CSIRO, North Ryde, N. S .W and Dr. J. L. Alcorn, Department of Primary Industry, Indooroopilly, Queensland (bryophyte isolate only). The results of the taxonomic identifications are shown in Table 3.1.

Table 3.1 Identification of Antarctic microfungi

Initial ID	Morphological ID	Source
Penicillium lake	*Penicillium expansum*	Windmill Islands
Penicillium sp2	*Penicillium hirsutum*	Windmill Islands
Penicillium sp17	*Penicillium commune*	Windmill Islands
Unidentified	*Embellisia* sp2. (see App III)	Bryophyte

No attempt was made to have the remaining fungal isolates formally identified based on their morphology.

3.1.2 Molecular identification

Genomic DNA was successfully extracted from each of the Antarctic and control (mesophilic) fungal isolates. PCR was performed amplifying the 18S gene for the Windmill Island isolates and the Marble Point *Embellisia* sp2. also the ITS region of all isolates. Results were confirmed by agarose gel electrophoresis by the presence of single bands within the expected size ranges (not shown).

3.1.2.1 RFLP analysis of the 18S gene and 18S sequence data

The RFLP analysis, performed with each of the Windmill Islands isolates, enabled a direct comparison to be made with control specie(s) of the same genus and some

outgroup species. Figure 3.2 illustrates the results obtained for the *Penicillia*, *Alternaria* and *Phoma* which (with the exception of *Phoma* sp.) appeared to confirm the tentative morphological identification of these isolates. The *Trichoderma* were analysed separately and produced similar confirmatory results (not shown).

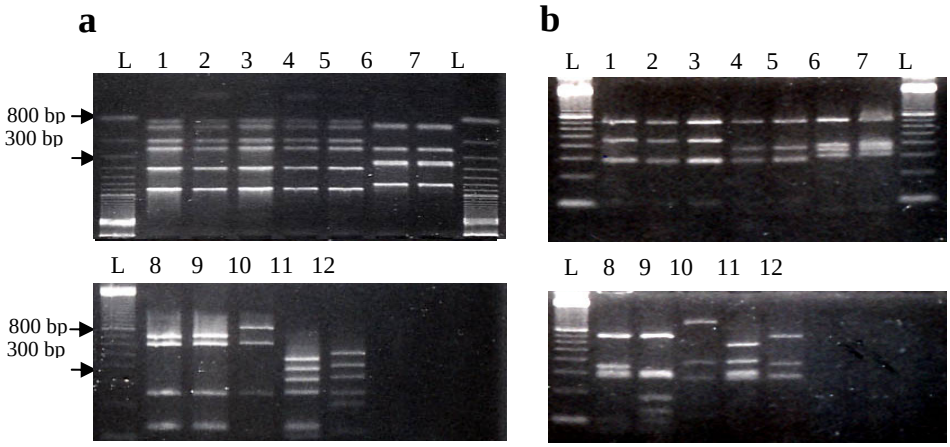

Figure 3.2 RFLP analysis of the 18S gene of Antarctic and control microfungal isolates with a) *Hin*f I and b) *Msp* I

L, 100 bp ladder; **1**, *P. expansum* (Antarctic); **2**, *P. hirsutum* (Antarctic); **3**, *P. commune* (Antarctic); **4**, *P. restrictum*; **5**, *P. decumbens*; **6**, *A. alternaria* (Antarctic); **7**, *A. alternaria*; **8**. *Phoma* sp. (Antarctic); **9**, *Phoma herbarum*; **10**, *Candida albicans*; **11**, *Rhizopus stolonifer*; **12**, *Aspergillus terreus*.

The 18S sequence data for each of the Windmill Islands isolates together with that of *Embellisia* sp2 recovered from the initial Marble Point bryophyte were displayed using the PRETTY program available through the ANGIS - Bioinformatic Service (http://morgan.angis.su.oz.au/new/bioinformatic/index.html). The pattern which emerged indicated that the sequences fell into three similar groupings (not shown).The first group included the three *Penicillia*, the second the two *Trichoderma* and the last, the dematiaceous (darkly pigmented) hyphomycetes *Phoma*, *Alternaria* and *Embellisia*. Each of the complete 18S sequences was compared with data present in the GenBank database available through National Centre for Biotechnology Information using the BLAST service (http://www.ncbi.nlm.nih.gov/BLAST/). Unfortunately, at this point in time, the number of complete 18S sequences deposited with GenBank is small resulting in an

inconclusive search in respect of the Antarctic microfungi. Sequence data has been deposited with GenBank adding considerably to the amount of data available at the time of submission. Details are provided in Table 3.2**.**

3.1.2.2 RFLP analysis of the ITS region

A RFLP analysis was only undertaken for the Windmill Islands isolates and the *Embellisia* sp2 (from the initial bryophyte). The digestion patterns which emerged with these isolates and the control species are shown in Figure 3.3 for the *Hin*f I digest. Once again the restriction pattern supports the morphological identification of the *Penicillia* (lanes 1-6), the *Trichoderma* (lanes 13-16) and the *Embellisia* (lanes 11- 12). However, the results obtained for the *Phoma* and the *Alternaria* are less supportive. It is notable that the restriction patterns which emerged for the control isolates, *Phoma herbarum* (lane 8), *Alternaria alternata* (lane10), *Embellisia* sp. (lane 12) and *Epicoccum nigrum* (lane 17) are virtually the same suggesting a close affinity between these genuses (see also App III). Similar results and conclusions could be drawn from the *Msp* I digest (not shown).

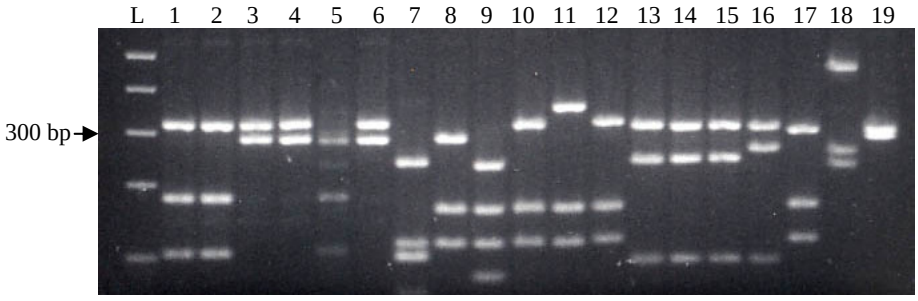

Figure 3.3 RFLP analysis of the ITS region of Antarctic and control microfungal isolates with *Hinf 1*

L, 100 bp ladder; **1**, *P. expansum* (Antarctic); **2**, *P. hirsutum* (Antarctic); **3**, *P. commune* (Antarctic);**4**, *P. decumbens*; **5**, *P. restrictum*; **6**, *P. janthinellum*; 7, *Phoma* sp. (Antarctic); **8,** *Phoma herbarum*; **9**, *A. alternaria* (Antarctic); **10**, *A. alternaria*; **11**, *Embellisia* sp2 (Antarctic); **12**, *Embellisia* sp.; **13**, *T. viride* (Antarctic);**14**, *T. koningii* (Antarctic); **15**, *T. viride*; **16**, *T. harzianum*; **17,** *Epicoccum nigum*; **18,** *Rhizopus stolonifer;* **19**, *Candida albicans.*

3.1.2.3 ITS sequence data of all Antarctic isolates

A BLAST analysis was performed on the ITS region (ITS 1 – 5.8S – ITS 2) sequence data obtained for all of the Antarctic isolates. This analysis confirmed the unique identity of each of the seven ornithogenic isolates from the Windmill Islands. In analysing the bryophyte isolates, nine different species were identified in this material. Although each of the bryophyte isolates has been designated to a particular genus and species derived from the best match in the GenBank database, there remain minor nucleotide variations between some isolates within the same classification which may indicate the presence of sub-species or additional species. As sequencing was performed directly from PCR products, the likelihood of these variations being due to PCR errors is reduced in view of the high copy number of nrDNA in the genome. A summary of the results of the BLAST identification are shown in Table 3.2.

With the exception of the *Phoma* sp2 and the isolate designated *Phaeosphaeria* sp., each of the other isolates bears an identity or close resemblance (≥ 98%) to species present in the broad mesophilic environment. Three of the genera identified here, *Ulocladium*, *Glomerella* and *Podospora*, do not appear to have been reported in association with bryophytes in previous Antarctic collections (Table 1.1) although, as has already been seen with the *Alternaria* and *Ulocladium*, identification based on morphological characteristics can lead to erroneous classification particularly with the dematiaceous hyphomycetes. The *Embellisia* has previously been reported as a new microfungal isolate from the Antarctic and likely a new fungal species (App III). The nucleotide variations which remain between the reference (GenBank) species and the recovered isolates and even between the isolates (1 - 3 nucleotides) may indicate a much wider range of speciation than immediately apparent.

Table 3.2 **Identification of Antarctic isolates based on ITS sequence data**

Morphological ID or Source ID (Refer to Table 2.1)	GenBank accession number [1]	Morphological ID or Source ID of other identical isolates (Table 2.1)	Closest Match in Database			Name allocated to species
			Accession No.	Species Name	% Identity[2]	
Penicillium expansum	AF218786		AJ005676	*Penicillium expansum*	100	*P. expansum*
Penicillium hirsutum	AF218787		AJ005484	*Penicillium allii*	100	*P. allii*
Penicillium commune	AF236103		AJ004813	*Penicillium commune*	100	*P. commune*
Trichoderma sp10	AF218790		X93984	*Trichoderma koningii*	100	*T. koningiii*
Trichoderma sp10-b	AF218788		X93979	*Trichoderma viride*	100	*T. viride*
Phoma sp2	AF218789		AF525674	*Setomelanomma holmii*	74	*Phoma* sp2
Alternaria alternata	AF218791		AF229488	*Ulocladium chararum*	100	*Ulocladium chararum*
Embellisia sp2	AF212307		AY278840	*Embellisia allii* [3]	98	*Embellisia* sp2
CC52/6G	AY345356	CP1/3a-2; CP1/3R; CC52/6a-2	AF212307	*Embellisia* sp2	100	*Embellisia* sp2
CC54/5a-1	AY345355	CC52/4b-1; CC54/5a-3				
C118/8R	AY345344		AJ301970	*Glomerella lagenaria*	99	*Glomerella lagenaria*
T489/9b-R	AY345345					
CC52/6a-1	AY345346	CC52/4a; CC54/5R	AF439507	*Phaeosphaeria triglochinicola*	93	*Phaeosphaeria* sp.
C239/10G	AY345347		AJ509866	*Geomyces pannorum*	98	*Geomyces pannorum*
T489/9b	AY345348	CC54/5a; CC54/5g;				
T557/7G	AY345349	CC54/5G; C239/10G; C239/10R C239/10a-1				

continues

Table 3.2 *continued*

T557/7a-1 T489/9a-R	AY345350 AY345351	T557/7b-1	AF443853	*Podospora minuta*	100	*Podospora minuta*
CC16/1b	AY345352		AF455525	*Cladosporium cladisporioides*	100	*Cladosporium cladisporioides*
CC16/1d	AY345353		AJ430405	*Helotiales* sp.	99	*Helotiales* sp.
T489/9a	AY345354		AF034457	*Penicillium corylophilum*	100	*Penicillium corylophilum*
CC54/5b	AY345356		AF033416	*Penicillium miczynskii*	100	*Penicillium miczynskii*

[1] Where isolates were obtained from the same source and have identical sequences, only a single representative accession has been lodged with GenBank

[2] The % Identity indicates how closely the isolate compares with its best match in the GenBank database.

[3] The 'Identity' details shown here refer to an entry made to the database in May 2003 for *Embellisia allii* (Pryor & Bigelow, 2003). Prior to this entry, the closest match was a *Pleospora* sp. with a 94% identity (see App III).

The use of molecular techniques for the taxonomic classification of fungal species has now become common practice and has also seen extensive application in the rationalisation of many fungal genera (Arenal *et al.*, 2000; Boysen *et al.*, 1996; Dodd *et al.*, 2000; Gams & Meyer, 1998; Kindermann *et al.*, 1998; Kuhls *et al.*, 1997; Lieckfeldt & Seifert, 2000; Pryor & Gilbertson, 2000; Skouboe *et al.*, 1999; Yao *et al.*, 1999). Often such studies result in a major reorganisation of the taxonomy based on prior morphological identification and classification. There is little doubt that the detailed molecular investigations undertaken by many researchers have aided greatly in clarifying fungal taxonomy. It would appear from some of the results obtained in this study that a general weakness remains in relying on information returned from GenBank enquiries as a definitive means of fungal identification. The problem would appear to stem from the submission of sequence data (particularly ITS) to the data bases and attributing it to a species previously identified using morphological techniques. If a large number of submissions are made for similar strains which have been misidentified, the reference databases are weighted towards the erroneous classification which can in turn influence subsequent lodgements, further compounding the problem. In Figure 3.4, an abridged taxonomic report returned by a BLAST enquiry for the Antarctic isolate recognised here as Podospora minuta is shown. This report demonstrates the wide variety of species identified as closely matching the ITS sequence data submitted (Figs. 3.5 & 3.6). Notwithstanding the small amount of variation present between the sequences held in the database (Figs. 3.5 & 3.6), the sources of the originating organisms not only crossed genera boundaries but were also attributed to differing orders, classes and even phyla.

Although there must remain some doubt as to the accuracy of the identification recorded above for each of the Antarctic isolates (Table 3.2) the fact remains that from a relatively

		BLAST Score	Identities
	Phylum		
	↓ Sub-Phylum		
	. ↓ Class		
	. . ↓ Sub-Class		
	. . . ↓ Order		
	. . . . ↓ Family		
	. . . . . ↓ Genus		
	. . . . . ↓ Species		
	. . . . . ↓		
	. . . . .		
	Ascomycota		
	. Pezizomycotina		
	. . Sordariomycetes		
	. . . Sordariomycetidae		
	. . . . Sordariales		
	. . . . . Lasiodphaeriaceae		
	. . . . . Podosporo		
1	. . . . . *Podospora minuta f. tetraspora* AF443853	920	464/464
	. . . . Diasporthales		
	. . . . . Valsaceae		
	. . . . . Phomopsis		
7	. . . . . *Phomopsis* sp. FP3-96 AF079773	795	451/465
10	. . . . . *Phomopsis* sp. BG-96 AF079771	787	450/465
	. . Sordariomycetes incertae sedis		
	. . . . Phyllachorales		
	. . . . . Phyllachoraceae		
	. . . . . Colletotrichum		
4	. . . . . *Colletotrichum truncatum* AF451907	827	454/465
	. . . . . Ascochyta		
3	. . . . . *Ascochyta lentis* AY131201	848	459/467
	. . Dothideomycetes		
	. . . . Pleosporales		
	. . . . . Pleosporaceae		
	. . . . . Leptosphaerulina		
5	. . . . . *Leptosphaerulina americana* AY278318	823	454/465
	. . . . . Ampelomyces		
9	. . . . . *Ampelomyces humuli* AF035779	795	451/465
11	. . . . . *Ampelomyces quercinus* AF035778	781	451/466
	. . . . . Epicoccum		
15	. . . . . *Epicoccum nigrum* AF455455	763	450/468
	. . Coelomycetes		
	. . . . Sphaeropsidales		
	. . . . . Phoma		
2	. . . . . *Phoma herbarum* AY293803	906	464/465
6	. . . . . *Phoma pinodella* AY131199	803	452/465
8	. . . . . *Phoma glomerata* AF126819	795	451/465
12	. . . . . *Phoma medicaginis* AF079775	779	449/465
	. . . . . Microsphaeropsis		
13	. . . . . *Microsphaeropsis amaranthi* AF079774	779	449/465
	Basidiomycota		
	. . Homobasidiomycetes		
	. . . . Hymenochaetales		
	. . . . . Hymenochaetaceae		
	. . . . . Phellinus		
14	. . . . . *Phellinus pectinatus* AY189705	763	450/468

Figure 3.4 BLAST taxonomy report for ITS sequence of *Podospora minuta* illustrating the range of species showing similar nucleotide sequences

The number appearing in the far left column is the order in which the sequences are arranged based on their identity scores.

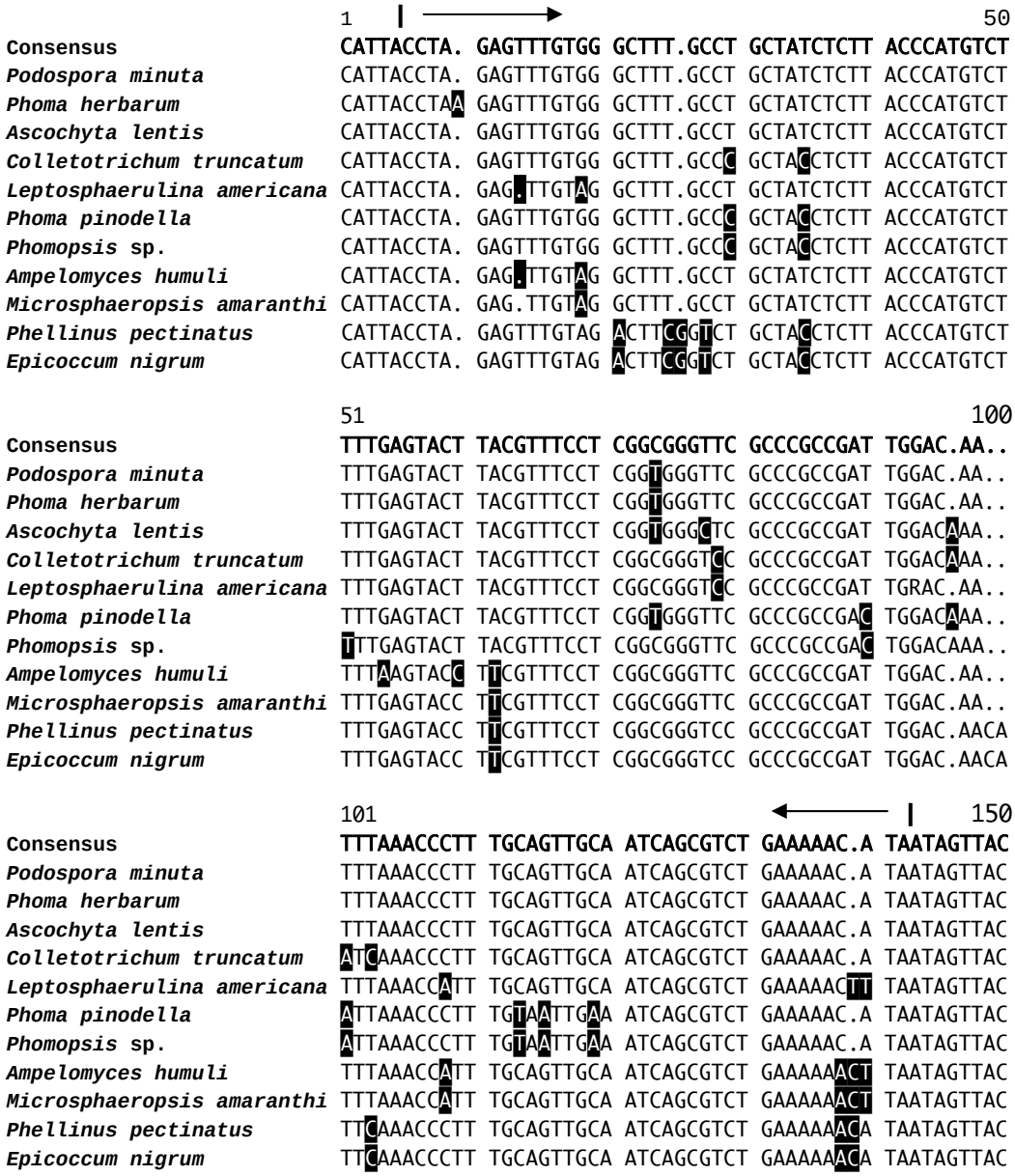

Figure 3.5 **ITS1 Sequence alignment of *Podospora minuta* showing variations from consensus in inverse font**

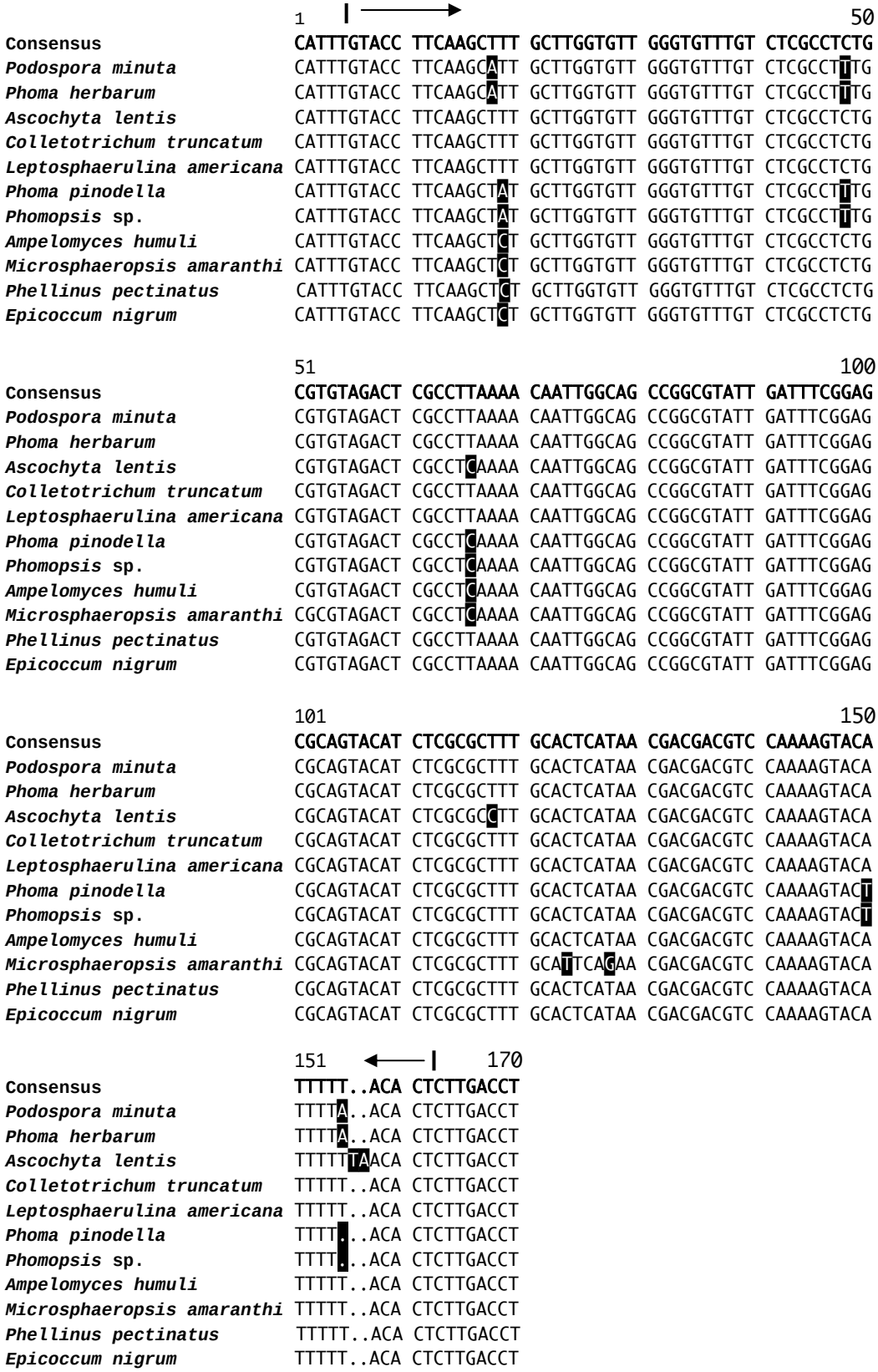

Figure 3.6 ITS2 Sequence alignment of *Podospora minuta* showing variations from consensus in inverse font

small and moderately diverse selection of source material a wide range of different microfungal isolates have been obtained. For the remainder of this study the identification attributed to each of the isolates (Table 3.2) will be used. From soil samples obtained within a single region and from eight bryophyte specimens from four neighbouring locations, not less than 14 unique microfungal species have been isolated. In the quest for a new source of microorganisms which may possess biotechnological potential, there appears to be an untapped wealth of new microfungi material present in the Antarctic environment.

3.2 Physiological factors affecting Antarctic microfungi

3.2.1 Trehalose production and its implications as a cryoprotectant

If an Antarctic microfungus utilises trehalose as a cryoprotectant or a major stress metabolite, it could be expected that the organism's metabolic machinery would be attuned to its production in response to cold induced stress. If such is the case, when the fungus is subjected to a cold environment, the level of production of this disaccharide could be expected to show a measurable increase above that of its mesophilic analogue under similar circumstances. To evaluate this hypothesis, the level of trehalose production of each of the selected Antarctic fungi and that of their best available mesophilic analogues were compared.

In every case it was found that the level of trehalose produced by fungi subjected to cold stress was significantly increased (44% - 154%) over that produced by unstressed fungi maintained at room temperature (Fig 3.7). Although the absolute level of trehalose production was increased in all isolates when faced with a cold induced stress, to establish whether or not the Antarctic species show a greater propensity to produce

trehalose is best assessed by comparing the change which occurs in the levels of trehalose produced.

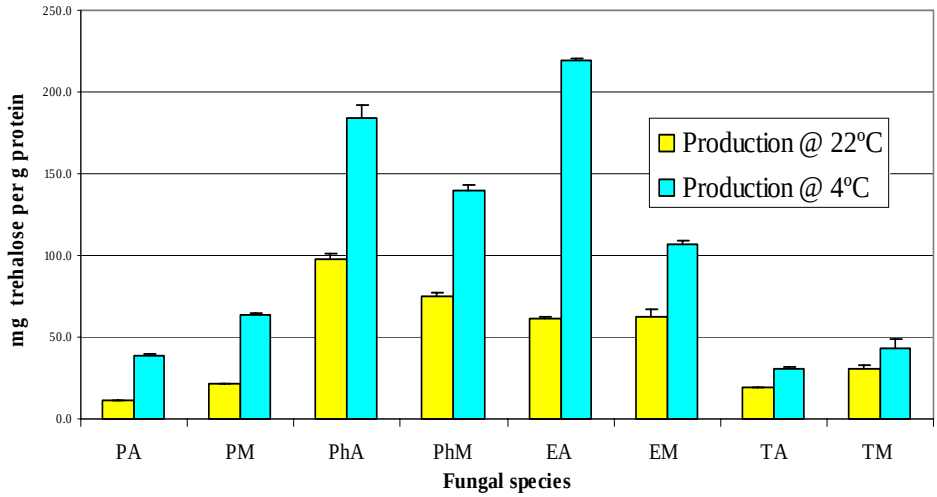

Figure 3.7 Trehalose production of selected Antarctic microfungi and their mesophilic analogues

PA, *P. expansum* (Antarctic) EA, *Embellisia* sp2 (Antarctic)
PM, *P. expansum* (mesophilic) EM, *Embellisia* sp. (mesophilic)
PhA, *Phoma* sp2 (Antarctic) TA, *T. viride* (Antarctic)
PhM, *Phoma herbarum* (mesophilic) TM, *T. viride* (mesophilic)
Error bars indicate S.E. of three assay replicates

In each of the fungal pairs examined, the per centage change in production by the Antarctic isolate exceeded that of its mesophilic counterpart (Fig 3.8). Only in the case of the *Embellisia*, with an increase in excess of 100%, can it be clearly demonstrated that trehalose production is significantly elevated in the Antarctic isolate. Although the result obtained for the *Penicillium* pair may be statistically significant, a difference of only 13.6% (± 3.1%) in the level of production (Fig 3.8) may not be biologically significant.

It would appear from the results obtained above that significantly increased trehalose production can be induced in both Antarctic and mesophilic microfungal strains by placing them under cold stress. A further conclusion that can be drawn from the data is

that althogh trehalose may be a factor, it is unlikely to represent the major cryoprotectant metabolite in Antarctic fungal species.

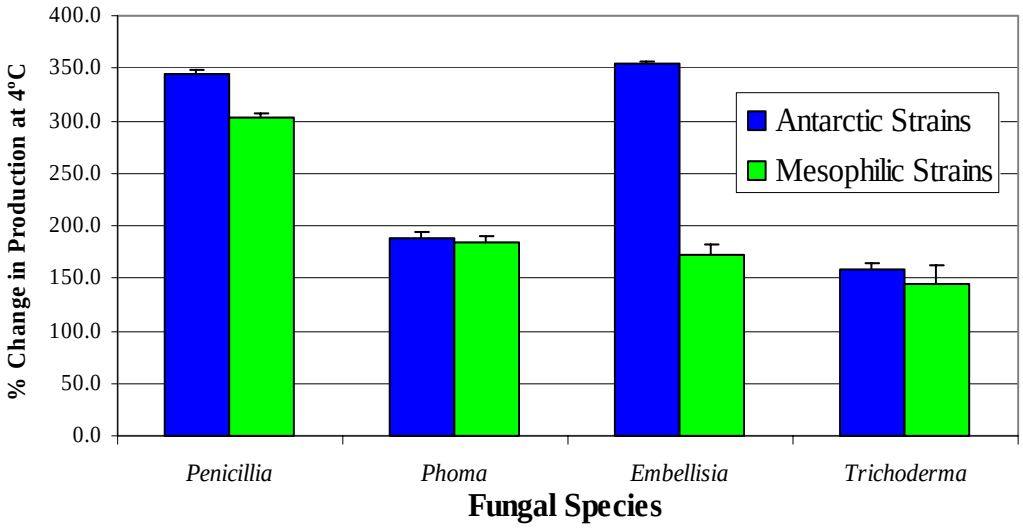

Figure 3.8 Change in trehalose production by microfungi grown at room temperature and under cold stess conditions at 4ºC
Error bars indicate S.E. of three assay replicates

The evidence indicates that trehalose may contribute significantly as a cold protectant of *Embellisia* sp2, although in general, it would seem that other compounds must be present which provide the protection necessary to ensure survival in the freezing conditions faced by Antarctic fungal species. The cryoprotectant capabilities of trehalose have already found industrial application as a preservative for such products as enzymes, foods, pharmaceuticals and cosmetics (Colaço *et al.*, 1992). The suggestion that Antarctic microfungi may produce other protective metabolites which fulfil a similar role to trehalose is a further indication of the potential worth of these microorganisms.

3.2.2 The potential of glycerol as a compatible solute to protect against osmotic stress

Mesophilic yeasts and fungi have been shown to accumulate glycerol as a compatible solute when subjected to osmotic stress, a condition likely to arise in the Antarctic

environment as a result of desiccation or elevated levels of salt. As outlined above (1.5.3), there is strong evidence supporting the presence of a shared stress response mechanism irrespective of the condition causing cellular stress. Specifically, cold stress conditions should induce osmotic stress which in turn will give rise to an increase in compatible solutes as a protective response as has been shown with *S. cerevisiae* (Lewis *et al.*, 1995; Schüller *et al.*, 1994).

Extending the principles outlined above for trehalose production (3.2.1), the same selection of Antarctic and mesophilic isolates were subjected to cold stress conditions and the level of glycerol production assayed and compared with that produced when the fungi have grown in an unstressed environment.

Unlike the outcome observed for trehalose production (Fig 3.7), no obvious pattern emerged for the production of glycerol by the fungi sourced from the different climatic regions when cultured under stressed and unstressed conditions (Fig 3.9). Glycerol production by the *Trichoderma* strains followed the predicted pattern: low levels of production in the unstressed growth environment but a substantial rise occurring when placed under stress (Fig 3.9). The actual levels of production by both of these isolates remained virtually the same under both growth conditions.

Glycerol production by both of the *Penicillium* isolates was relatively high in the unstressed condition, with the Antarctic isolate exceeding the mesophilic output by 53% (Fig 3.9). However, under the effects of cold stress, production by the mesophilic isolate remained virtually unchanged whereas, contrary to prediction, glycerol production by the Antarctic isolate was suppressed and fell by 78% (Fig 3.9). The *Embellisia* isolates produced very low levels of glycerol and the effects of cold stress were indeterminate and

for the *Phoma* isolates production was virtually non-existent under any conditions (Fig 3.9).

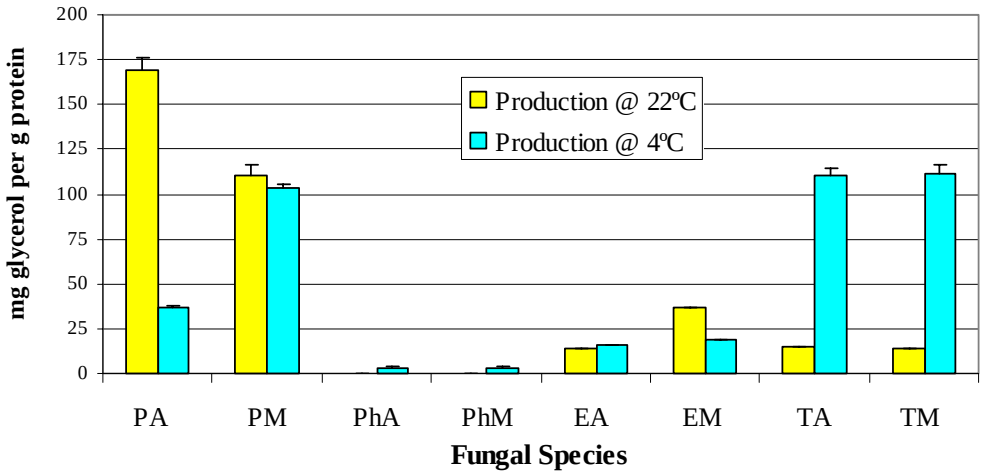

Figure 3.9 Glycerol production of selected Antarctic microfungi and their mesophilic analogues
For Fungal Species legend refer to Fig 3.7
Error bars indicate S.E. of three assay replicates.

With the exception of the *Trichoderma*, overall results do not provide support for glycerol acting as a compatible solute for Antarctic microfungi. This conclusion can be contrasted with glycerol's role as one of the principle agents employed by mesophilic fungi and yeasts in response to osmotic shock (Foerster *et al.*, 1998; Hazell, 1999; Pascual *et al.*, 2000). In the absence of glycerol providing protection against osmotic stress in Antarctic fungi some other compounds must fulfil this role.

When considering Antarctic microfungi as a potential bioresource, compounds identified in other extremophilic organisms which accomplish the same functions can act as a guide. Generally, the research and literature have focused on thermophilic Archaea and Bacteria (Santos, 2000). A number of novel low molecular weight organic compounds have been identified from these sources, for example, ectoine and hydroectoine

(tetrahydropyrimidine derivatives), Firoin (mannosylglycerate), Firoin A (mannosylglyceramide), DIP (di-*myo*-inositol phosphate), cDPG (cyclic diphosphoglycerate) and DPG (diglycerol phosphate) (see http://www.bitop.de and the references provided therein). Some of these have already found commercial applications in a range of skin protection and care products. It could therefore be extrapolated that a corresponding group of compounds exist which provide a similar level of protection to psychrophilic and psychrotolerent organisms, particularly the Antarctic microfungi.

3.2.3 Oxidative stress response in Antarctic microfungi

The dihydroethidium (DHE) stain which was introduced to the mycelial samples of each of the fungal isolates is known to react in the presence of ROS. This stain is a phenanthridinium dye structurally similar to ethidium bromide and propidium iodide but unlike these last two, it is cell permeant. In the presence of ROS in viable cells, DHE is oxidised to ethidium which intercalates with the DNA and when illuminated by light with a wavelength of 488 nm, will fluoresce. The greater the level of ROS present in the cells, the greater will be the level of fluorescence that can be detected. Conversely, the better adapted an organism is at combating the effects of ROS through the production of antioxidants, the lower will be the level of ROS in the cell. This in turn will result in reduced levels of detectible fluorescence in the presence of DHE.

Antarctic microfungi can be subjected to high levels of oxidative stress due to the extreme growth conditions they face in their native environment (1.5.5). As such it could be expected that their ability to guard against the potential damage wrought by ROS would be enhanced above that of their mesophilic counterparts. Thus, in a controlled analysis of paired strains of microfungi obtained from mesophilic and Antarctic sources, the Antarctic isolate could be expected to develop higher levels of protective antioxidants

when subjected to stress than its mesophilic counterpart which, in the presence of DHE, would result in lower levels of fluorescence.

3.2.3.1 Qualitative evaluation

This examination of the Antarctic and mesophilic fungal strains was undertaken to establish firstly, if the level of fluorescence generated by the reduction of DHE to ethidium and its intercalation with the DNA was at sufficiently high enough levels as to be detectible and recordable under fluorescent microscopy. Assuming a positive result in this first step, was there an observable difference between the levels of fluorescence obtained under the different stress and non-stress conditions and between the Antarctic and mesophilic pairs.

In Figure 3.10, the photographic results of the fluoro microscopic analysis are shown. It is quite apparent that there is sufficient chemical reactivity with the DHE in these well developed mycelia to produce measurable levels of fluorescence. It is however difficult to discriminate between the fluorescence emitted from the Antarctic and mesophilic isolates or to discern differences in the level of fluorescence between the stressed and unstressed conditions. As the level of fluorescence recorded in any of the examinations can be affected by such simple variables as the film exposure time or the quantity and density of the mycelial material present under the objective lens of the microscope, any interpretation of the activity recorded can be only subjective at best.

3.2.3.2 Quantitative evaluation using flow cytometry

In order to overcome the deficiencies identified in the fluorescence microscopy analysis, an alternative method was sought which would allow the level of fluorescent activity arising from the interaction of the dye with the ROS to be quantified. One method of

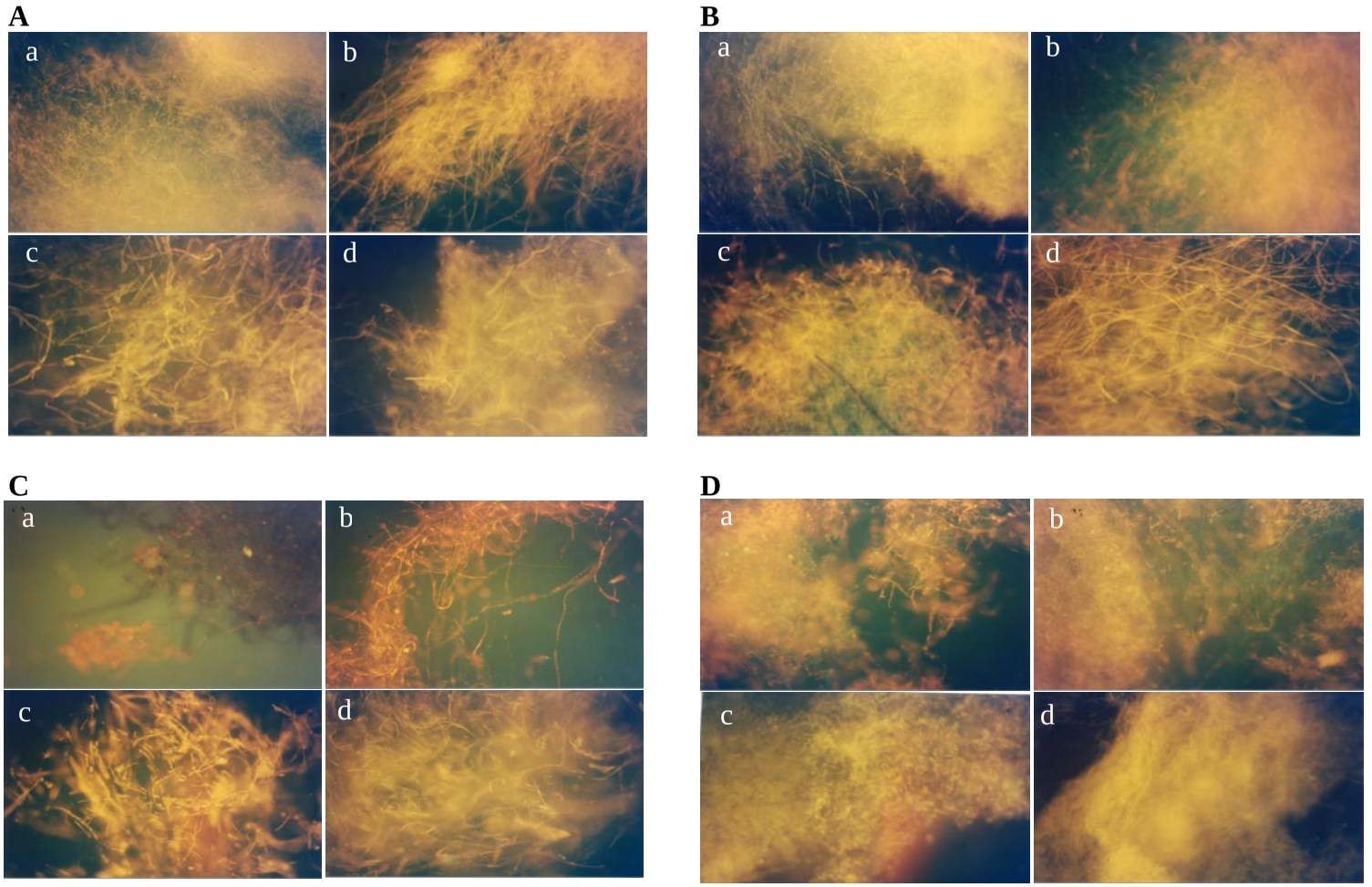

Figure 3.10 Antarctic and mesophilic fungal isolates stained with dihydroethidium and viewed by fluorescent microscopy (488 nm, x200)

Panel A *Penicillia*; Panel B *Embellisia*; Panel C *Phoma*; Panel D *Trichoderma*

a Antarctic isolate @ R/T; **b** mesophilic isolate @ R/T; **c** Antarctic isolate @ 5ºC; **d** mesophilic isolate @ 5ºC

quantification which has been used extensively for bacteria and yeast in similar circumstances is flow cytometry (FC). Although FC has seen limited application in the study of fungal spores, no literature was available describing its application to the study of filamentous fungi at germination and in the early stages of hyphal development (1.6.2).

Extensive experimental work was undertaken to established the practicality of applying FC to fungal analysis, described in detail in Appendix IV. It was found that there was a period of time, variable between species, during which FC could be used to track the development of filamentous fungi from germination to the development of hypha. Not only could the physical growth changes be measured using the forward (FSC) and side scatter (SSC) detectors (see Fig 1/App IV) but with the introduction of permeant fluorescent dyes, physiological activity could also be evaluated using the fluorescence detectors (see Fig 2/App IV).

In the course of evaluating physiological activity with the FC, two fluorescent dyes were assessed, DHE which was used in the qualitative assessment (3.2.3.1) and hexidium iodide (HI) a similar phenanthridinium dye. It was found that the HI was far more sensitive to the oxidative action of ROS than the DHE (see Table 3/Ann IV and Fig 2/Ann IV). During the evaluation, the fungal strains were placed under mild stress and the resultant oxidative stress induced was found to produce a quantifiable variation in the detectors with DHE as the stain.

Quantification of the oxidative activity exhibited by the selected Antarctic and mesophilic microfungal isolates was attempted using DHE as the fluorescent dye and incubations were again at R/T and 5ºC in liquid medium inoculated with spores. It was found that the germination and development of spores in the culture medium at 5ºC

proved to be erratic. Unlike the development pattern which prevailed at higher temperatures (R/T) where spores developed fairly uniform cohort, at 5ºC development occurred over an extended time frame. When analysed by FC, the latter produced a broad spread of activity which defied meaningful analysis.

Further examination has indicated that more uniform development of the microfungal spores can be obtained if the incubation temperature is marginally increased to about 10ºC. Although the results obtained for this phase of the investigation remain inconclusive, the results previously obtained when developing the technique (App IV) would indicate that, given the right experimental conditions, FC would permit the level of oxidative activity present in the various developing microfungi to be quantified and compared.

3.3 Activity in Antarctic microfungi when grown on solid media

3.3.1 Growth patterns at low temperature

The microfungi recovered from the bryophytes were cultured on standard plates containing PDA at 10ºC and at room temperature (22-23ºC) in order to gain some measure of their growth pattern. The results obtained (Table 3.3) showed that each isolate grew successfully at both temperatures. The *Embellisia* sp2 was observed to grow particularly vigorously under each of the temperatures regimes and growth by *Cladosporium cladosporioides* at 10ºC exceeded that attained at R/T. For comparison, two mesophilic species, *Trichoderma viride* and *Phoma herbarum* were also grown under identical conditions. The *Trichoderma* when grown at R/T covered the plate (85 mm) after 4 days and at 10ºC after 7 days. It took the *Phoma* 15 days to cover the plate at R/T by which time diameter on the 10ºC plate had reached 55 mm.

Table 3.3 Growth of Antarctic microfungi at 10ºC and room temperature on PDA plates

Antarctic species	Incubation Temperature	Colony diameter (mm) Number of days			
		5	**10**	**15**	**20**
Embellisia sp2.	10º	17	40	57	75
	R/T	29	63	85*	
Cladosporium cladosporioides	10ºC	7	15	22	29
	R/T	6.5	12	19	22
Helotiales sp.	10ºC	6	13	17	25
	R/T	5	14	22	na
Phaeosphaeria sp.	10ºC	10	16	20.5	28
	R/T	10	20	32	38
Geomyces pannorum	10ºC	12.5	27	37	52
	R/T	9	29	62	73
Podospora minuta	10ºC	13	25	31	36
	R/T	23	36	47	54
Penicillium corylophilum	10ºC	11	29	32	45
	R/T	23	na	na	na
Glomerella lagenaria	10ºC	5	9	12.5	17
	R/T	7	14	24	28
Penicillium miczynskii	10ºC	5	17	26	31
	R/T	na	na	na	na

Experiments were performed in triplicate using plugs of a fixed size cut from a growing colony.

* limit of standard plate

na not available

3.3.2 Qualitative assessment of hydrolytic activity over a range of temperatures

The growth substrates chosen for this study represent organic polymers that are potentially available to microfungi in the Antarctic. Typically, organic matter may originate from sediments, bird colonies and research bases. The ability of a fungus to grow on a particular polymer indicates the production and secretion of enzymes that degrade that polymer, producing smaller molecules that can be taken up and metabolized by the organism. In many cases, several enzyme activities are required for the hydrolysis of polymeric substrates. Formation of a clearing halo around the growing fungal colony on a screening plate would thus indicate a concerted synthesis, secretion and activity of appropriate enzymes at a given temperature.

Some of the results obtained for this part of the study are presented in Appendix I.

3.3.2.1 Growth of fungal colonies on different carbon sources

The diameter of fungal colonies attained after seven days' incubation at each temperature and for each of the selected carbon sources are shown in Table 3.4. With the exception of *Embellisia* sp2, other bryophytic fungi were only assessed for their activity on plates containing locust bean gum and birchwood xylan. The first signs of growth were usually visible by the second day for all carbon sources when incubated at 21ºC or 28ºC. For incubations at 10ºC and 37ºC growth, when it occurred, was usually visible by the third day on glucose-skim milk, pectin and PDA plates. On other substrates, growth was not apparent until the fourth or fifth day. Growth was recorded as 0 if either no growth could be observed or colony size was less than 0.5 mm. All fungal strains were found to grow well at mesophilic temperatures of 21^{o}C and 28^{o}C regardless of the carbon source supplied in the growth medium with the following exceptions. The *Phoma* sp2 displayed an inability to degrade cellulose at any temperature and *Podospora minuta* and *Geomyces pannorum* displayed limited or no ability to degrade the birchwood xylan (Table 3.4). In general, colony sizes, if growth occurred, were about 50-75% smaller at the extreme temperatures (10ºC and 37ºC) on each substrate.

Growth of the isolates at different temperatures on PDA, a rich medium, followed the same general pattern as that obtained for the other selected substrates. On the whole, colony sizes on PDA plates were somewhat larger than those obtained on the corresponding plates utilizing a single carbon source particularly at 21^{o}C and 28^{o}C with the notable exception of *Penicillium commune* (Table 3.4). Interestingly, the optimum growth temperature on PDA and a single carbon source plate did not always coincide.

Table 3.4 Colony growth (diameter in mm) of Antarctic microfungi cultured on a range of sole carbon sources at varying temperatures

A.

Carbon source	Temperature °C	*Penicillium expansum*	*Penicillium allii*	*Penicillium commune*	*Ulocladium chararum*	*Phoma* sp2	*Embellisia* sp2	*Trichoderma koningii*	*Trichoderma viride*
Glucose &	10	7.5	6.5	7	4.5	0	2	6	5
Skim milk	21	20	18	15	10	5	9	25	20
	28	20	20	10	9.5	6	11	16	16
	37	0	0	0	10	5	0	7	6
Olive oil	10	6	0	5	4	0	0	4	2.5
	21	14	8	10	5	4.5	4	17	26
	28	14	8	8	8	8	9	18	30
	37	0	0	0	0	0	0	0	0
Walseth Cellulose or	10	0	0	0	0	0	2	9	3
Hydroxyethylcellulose	21	10	5	9	6	0	6	18	18
(See footnote)	28	10	8	8	9	0	7	18	22
	37	0	0	0	6	0	0	0	0
Locust bean gum	10	6	5	5.5	0	0	2.5	7.5	7
	21	15	11	11.5	6	6	6	14	21
	28	13	9	9.5	7	5	7	18	25
	37	0	0	0	2	0	0	0	0
Birch xylan	10	4	0	4	3.5	0	1.5	4	4
	21	12	9	9	4	5	5.5	15	18
	28	15	9	7	6	5	6	14	19
	37	0	0	0	3.5	0	0	0	0
AZCL-Pachyman	10	0	0	4	5	0	2	5	4
	21	12	9	8	5	4	4.5	14	17
	28	10	8	6	8	4.5	6	12	18
	37	0	0	0	0	0	0	0	0
Chitin	10	4	3	4	0	0	1.5	6	4.5
	21	6	8	9	5	4	4	10	17
	28	6	9	7	6.5	4.5	3	12	6
	37	0	0	0	0	0	0	0	0
Pectin	10	7	3	4	3	2	1.5	5	5
	21	13	10.5	8	5	5	6	13	19
	28	14	11	6	7	6	7.5	13	20
	37	2	0	0	4	0.5	0	0	2
PDA	10	5	3	1	5	1	3	6	5
	21	19	13	8	10	8	9	19	25
	28	15	10	6.5	11	7	12	13	13
	37	1.5	1.5	0	2.5	0	0	2	2

Plates were incubated for 7 days at each temperature. 0 = no growth or growth < 0.5 mm. Numbers given are averaged from four replicates of each strain. Variation between replicates ≤ 5%.

Hydroxyethylcellulose was used in place of Walseth cellulose as the carbon source for *Embellisia* sp2 only.

Table 3.4 *continued*

B.

Carbon source	**Temperature °C**	*Cladosporium cladosporioides*	*Phaeosphaeria* sp.	*Geomyces pannorum*	*Podospora minuta*	*Penicillium corylophilum*	*Glomerella lagenaria*
Locust bean gum	10	3.5	1.5	0	0	4	3
	21	5	4	6	9	6	5
	28	1	2.5	9	7	7	1
	37	0.5	0	0	0	1	0
Birch xylan	10	2.5	1	0	0	2	2.5
	21	6	2	6	0	9	4
	28	1	2.5	0	0	10	3
	37	0	0	0	0	0	0

For example, both *Phoma* sp2 and *Trichoderma viride* displayed optimum growth on the olive oil plates at 28ºC whereas maximum growth was obtained at 21ºC on the PDA plates.

Among all isolates, growth occurred more frequently at 10ºC than at 37ºC (Table 3.4) as may be expected for Antarctic microfungi. Small colony sizes after seven days' cultivation at 10ºC are not surprising, assuming that the fungal metabolism would be slowed down at this temperature. The effect of a longer cultivation time on growth at 10ºC was tested with birch xylan plates which were incubated for an additional week after initial scoring. Measurements taken after incubation for two weeks (not shown) indicated that significant further growth did occur, seen by a more than doubling of the colony size in *Penicillium* and *Trichoderma* isolates. This observation warrants further investigation into the hydrolase activities from Antarctic microfungi as a function of temperature in the search for novel cold active enzymes.

Of the eight fungal isolates cultured on all of the substrates, *Ulocladium chararum* indicated its adaptability to the whole range of growth temperatures by being able to utilise most of the carbon sources surveyed. Vigorous colony growth, comparable to that at mesophilic temperatures was recorded on protease screening plates at 37ºC. *Trichoderma* spp. proved the most versatile of the species examined. Both isolates were capable of metabolizing all the substrates tested and exhibited aggressive growth behaviour, particularly in the intermediate temperature range (Table 3.4).

3.3.2.2 Relative enzyme activity levels

For a fungus to successfully utilise a polymeric carbon source it must secrete hydrolytic enzymes at least to some extent. This hydrolase activity is often referred to by its generic name. For example, the hydrolysis (clearing) of Walseth cellulose would involve three different types of enzymes, collectively identified as “cellulase” activity. Thus, the plate screening techniques applied in this study would give an indication of the overall ability of a fungal isolate to hydrolyse a given substrate at a given temperature but does not provide detailed information on the performance of different individual enzyme components involved in the hydrolysis. Other factors affecting the formation of clearing halos on plate cultures are the diffusibility and activity of enzymes produced, and concentration of the carbon source.

Although this investigation is examining microfungi isolated from the Antarctic, in addition to assessing the level of enzymatic activity at 10ºC it has also been assessed at 21º and 28ºC, a mesophilic growth range and at 37ºC which approaches the maximum temperature for growth of the fungal isolates. Although these mid-range temperatures are most conducive to the growth of mesophilic species their maximum enzyme activity generally occurs at much higher temperatures whereas high levels of activity in this range

can be indicative of cold-active enzymes (see Brenchley, 1996; Ohgiya *et al*., 1999 and Fig 1.6).

Figures 3.11A, B, C and E, which were included in Appendix I (see Fig 1/App I), have been reincluded here for completeness. They have been updated to reflect the identification of the isolates (refer Table 3.2) and now also include details for the bryophytic microfungus *Embellisia* sp2.

A gross measure of the level of production of enzymes for the degradation of a given substrate is provided by the index of relative activity, RA, introduced in Materials and Methods (2.4.1). A high relative activity index is an indication that the fungus is secreting enzyme(s) into the growth medium at a rate greater than it can utilise it for its immediate growth. In Figure 3.11A-H this relative activity is illustrated for each substrate and growth temperature.

Of the enzymes studied, the proteases and the hemicellulases (xylanase and β-mannanase), were most effectively secreted into the growth environment by all isolates across the range of cultivation temperatures (Fig 3.11A, B and C) indicating the general importance of these enzymes in probing for nutrients. A relatively high amount of active acid protease is secreted by *Trichoderma viride* at 10ºC (Fig 3.11A) indicating that this isolate may have developed some cold-tolerant properties. Worth particular note is the high hemicellulase activity indices across the range of temperatures exhibited by *Embellisia* sp2 (Fig 3.11B and C). The extremely high values obtained for activity at 10ºC for both xylanase (RA index 127.4) and β-mannanase (RA 24.0) suggests the likelihood that this isolate is either indigenous to the Antarctic or at least well adapted to

the cold. The high RA values for the hemicellulases exhibited by the *Ulocladium chararum* (shown as *Alternaria alternata* in Fig 1/App 1) isolate at 37ºC (Fig 3.11B and C), especially for β-mannanase (RA index 55), suggests a potential for this species to respond to higher growth temperatures.

Production of lipase was clearly highest at moderate temperatures for the *Penicillia*, *Ulocladium* and the *Phoma* (Fig 3.11D). Notwithstanding the small to non-existent RA indices determined for the *Embellisia* and *Trichoderma*, each displayed high levels of growth at the intermediate temperatures (Table 3.4) suggesting that these isolates were actively secreting lipase but only at a rate sufficient to meet the requirements for growth.

At the low end of the temperature range, chitinase production by the *Penicillium* and *Trichoderma* isolates seemed to be effective at 10ºC (Fig 3.11E) suggesting that the degradation of chitin at a low temperature may be essential for these fungi. This view is supported by the fact that many of the fungal isolates were collected from ornithogenic soils, which very probably contain high amounts of the remains of crustaceans (krill) which represent the principal food source of penguins. In contrast, the *Embellisia* which was recovered from a bryophyte displayed no discernible chitinolytic activity. Complete degradation of chitin by "chitinase" involves three different types of enzyme activities, all of which have been shown to be produced by *T. harzianum* (Harman *et al.*, 1993). These enzymes have, in general, been shown to be active at mesophilic temperatures which can be supported by the results of this study (Fig 3.11E). The relatively high level of activity at 10^{o}C in the Antarctic *Trichoderma* isolates strongly suggests a true cold adaptation for chitin degradation. In *Penicillium allii* and the *Trichoderma* isolates the strong chitinolytic activity found both at 10ºC and 28ºC (Fig 3.11E) points to the

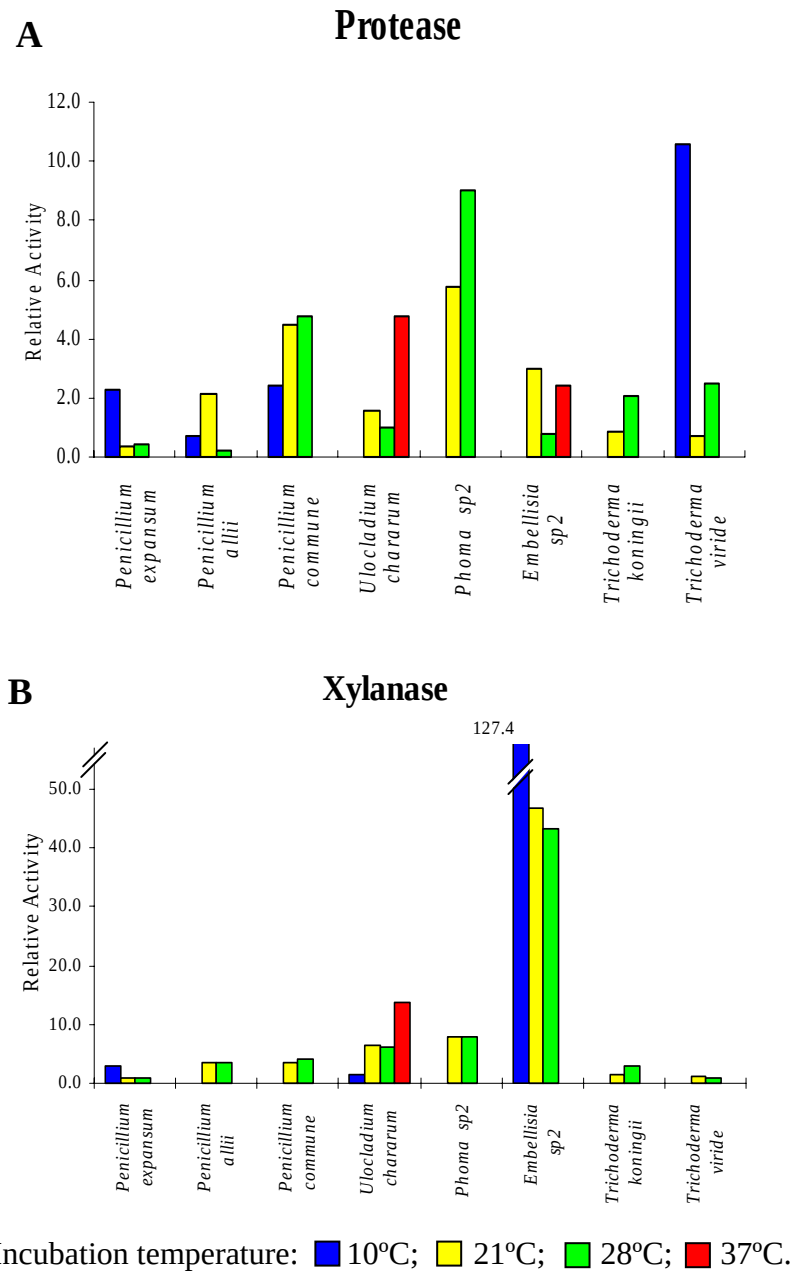

Figure 3.11 Relative enzyme activity displayed by Antarctic microfungi incubated at different temperatures on selected substrates

Relative activity was determined from the average values of four replicates and the method of calculation together with the substrates used are described in Materials and Methods 2.4.1.

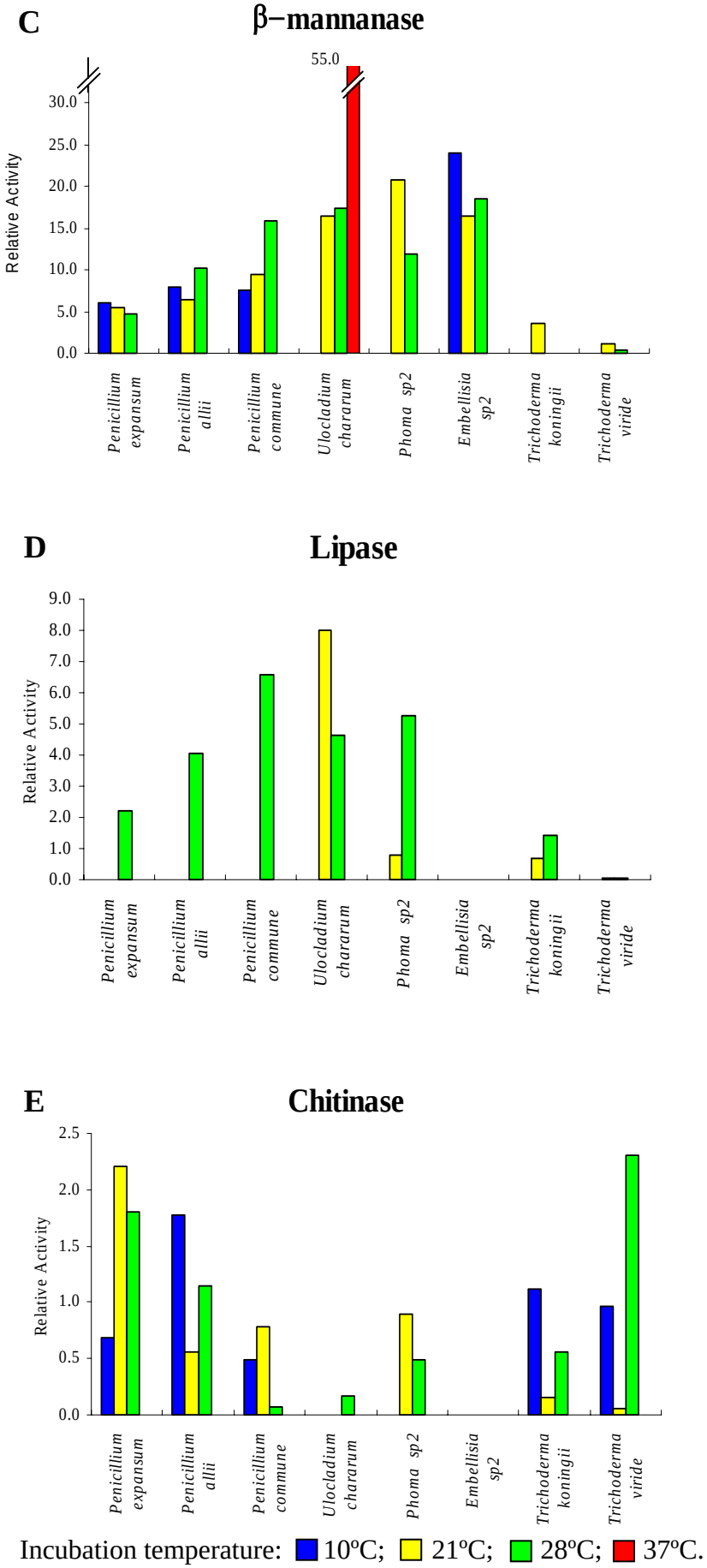

Figure 3.11 *continued*

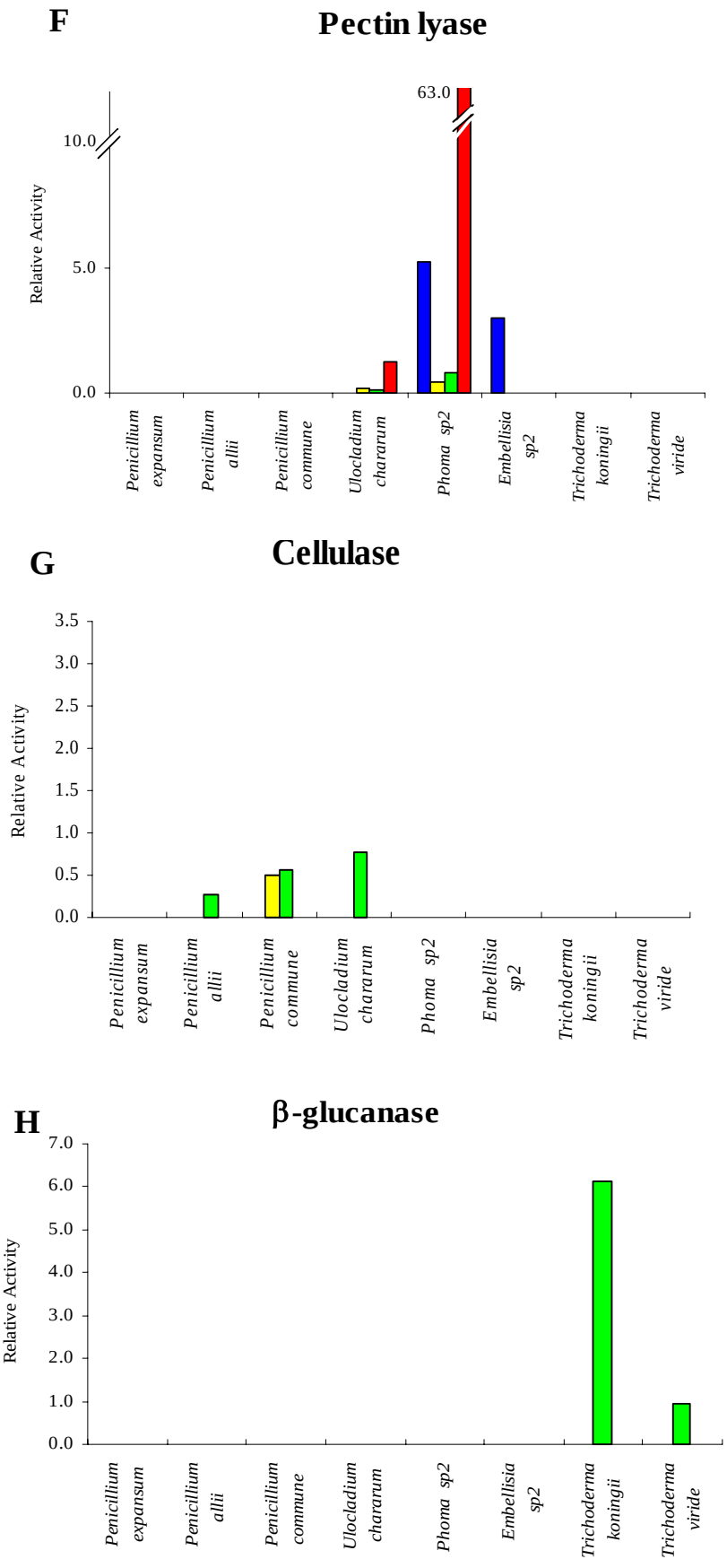

Figure 3.11 *continued*

possibility that different sets of enzymes are synthesized to accommodate different temperature regimes.

The RA values for fungi cultivated on cellulose and β-glucan were in general low, suggesting a direct use of these substrates to support effective biomass production (Fig 22G and H). The most striking example of the opposite characteristic, high enzyme activity produced by a small colony, is provided by pectin lyase in *Phoma* sp2 at 37^{o}C (Fig 3.12F) with a calculated RA index number of 63 compared with 5.5 at 10^{o}C and 0.8 at 28^{o}C, notwithstanding a very small colony size at 37^{o}C (see Table 3.4). Although colony formation occurred on plates containing pectin (Table 3.4) only *Ulocladium chararum* and *Phoma* sp2 showed the production of pectin lyase (Fig 3.12F). The ruthenium red staining as described in Materials and Methods, indicated the presence of pectin methyl esterase in *Penicillium allii* at 10ºC, *Ulocladium chararum* at 10ºC and 37ºC, and *Phoma* sp2 at all temperatures (not shown).

In summary, the Antarctic microfungal isolates studied showed general growth characteristics and hydrolase production resembling that of mesophilic species. However, some strains and enzymes showed adaptation to elevated and/or colder temperatures indicating the development of means to deal with environmental stress generated by non-mesophilic growth temperatures. The relatively good growth and activity displayed by a number of isolates at 10ºC indicate the potential of Antarctic isolates as a source of novel cold-active enzymes.

3.4 Characterisation of hemicellulases from selected Antarctic microfungi

The hemicellulases secreted into the growth medium by a selection of Antarctic microfungi from the Windmill Islands' collection was characterised in detail. Both mannanase (*endo*-β-1,4-mannanase; E.C. 3.2.1.78) and xylanase (*endo*-β-1,4-xylanase; E.C. 3.2.1.8) activity were assayed over a series of temperatures rising in 10ºC steps from 0ºC to 80ºC across a pH range from 2 to 11. For comparison, a mesophilic *Trichoderma reesei*, recognised as a high producer of hydrolases, was assayed under identical conditions. Full details of the results are provided in Appendix II and are summarised below.

There is considerable potential for cold acting hemicellulases in industrial processes where there is a need to maintain a low temperature environment. This is particularly true for food processing where it is essential to retain flavours and avoid product denaturation due to heat such as in the fruit-juice extraction industry. The presence in a number of Antarctic isolates of broad range of both temperature and pH over which high levels of enzymatic activity is maintained offers even greater potential for exploitation.

It is unfortunate that at the time of undertaking this phase of the study, the *Embellisia* sp2 isolate had not been discovered. In view of the high levels and temperature range for which both mannanase and xylanase activity were identified when assayed on solid media containing single carbon sources (refer 3.3.2.2), inclusion of this isolate could have been very informative.

When referring to Appendix II, the change of identity based on the molecular identification (see 3.1.2.3) of *Penicillium hirsutum* to *Penicillium allii*, and *Alternaria alternata* to *Ulocladium chararum* should be noted.

3.4.1 Mannanase activity

The reference *T. reesei* was found to display a classical activity profile with temperature change, rising regularly from a low levels, 15% of maximum at 30ºC, to reach a peak slightly above 70ºC then declining rapidly as temperature increased further (Fig 1b/App II). In contrast, each of the Antarctic isolates produced its maximum level of activity significantly below that of the mesophilic reference species, from as low as 40ºC for *P. expansum* and *P. allii* ranging to 60ºC for *U. chararum* (Fig 1/App II). Possibly more significant, for all of the Antarctic isolates except *U. chararum*, activity exceeded 50% of their maximum over a 40º – 65ºC temperature range compared with only a 30ºC range for *T. reesei*. The P. expansum isolate was even able to maintain 50% of its maximum activity at 0ºC and the *Phoma* sp2, 40% at freezing point.

There was no appreciable difference observed between the reference and Antarctic strains for the pH level at which maximum activity occurred (pH 3 – 4). However as with temperature profile, it was found that activity in all of the Antarctic isolates, with the exception on this occasion the *P. allii*, activity remained above 50% of maximum over the whole pH range assayed (Fig 2/App II). It was also observed that additional peaks at higher pH values were present in this profile suggesting the possibility of more than one mannanase being secreted.

3.4.2 Xylanase activity

The activity profile of the Antarctic isolates showed many of the features already observed with the mannanase. Where *T. reesei* produced its maximum activity at 60ºC, shared by two of the *Penicillia*, *P. expansum* and *P. allii*, the remainder had their maximum 10º – 20ºC lower (Fig 3/App II). Once again the activity tended to extend over

a broader temperature range than was apparent for the reference strain however not nearly as marked as with mannanase.

For all isolates, activity in excess of 40% was maintained across the pH range, in sharp contrast to *T. reesei* which fell away sharply to 0% at pH 8 after reaching a maximum at pH 4. This maximum activity at pH 4 – 5 was shared by all of the isolates (Fig4/App II).

3.5 Cloning of an Antarctic *Penicillium allii* lipase gene and expression in *Trichoderma reesei*

The opportunity existed to investigate the Antarctic microfungal species for potential cold-active enzymes and isolate their genes directly from the gDNA. Using a suitable fungal host as a production platform and the biolistic transformation system, a gene could be integrated into the heterologous host and the resulting transformant induced to secrete an active protein into the growth medium.

When screening the Antarctic microfungi for the production of hydrolytic enzymes using single carbon sources (3.2.2), significant lipase activity was identified on the plate cultures containing olive oil and rhodamine B (Fig 3.11D) in the temperature range 21º – 28ºC. As previously indicated (3.3.2.2), evidence of enzyme activity in this range can be indicative of the presence of cold-active enzymes (see Fig 1.6). Lipases have found a wide range of commercial uses and in particular, high value applications in the chemico-pharmaceutical industries (1.7.4.4). For these reasons, lipase was targeted as attractive candidate for this phase of the study.

The results of this investigation are included in Appendix V, and are summarised below. Additional material, not included in Appendix V, has been incorporated into the following discussion.

3.5.1 Isolation and identification of lipase gene

Lipases are serine hydrolases with an active site formed by a catalytic triad consisting of serine, a carboxylic acid and a histidine residue (Brzozowski *et al.*, 2000; Jaeger *et al.*, 1994). Investigations into the amino acid sequences of bacterial and fungal lipases have identified the region in the vicinity of the active-site serine and a second region corresponding to the oxyanion hole (Herggård *et al.*, 2000; Jaeger *et al.*, 1994) to be relatively well conserved. These two sites, which in filamentous fungi appear to be consistently separated by around 72 aa (216 bp), were targeted as the most promising for the design of PCR primers. Due to the significantly reduced level of homology that existed at the nucleotide level, a set of degenerate PCR primers was designed using the CODEHOP design principle (Rose *et al.*, 1998).

The PCR primer set was applied to the gDNA of a number of the promising lipase producing isolates (see Fig 3.11D). Early success was achieved in obtaining bands of the expected size (App V) using the *P. allii* gDNA. When sequenced and translated to amino acids, a sequence was obtained which displayed good homology with known lipases. Genomic-walking PCR (Morris *et al.*, 1995; 1998) was used to obtain a full length gene sequence which was designated *lipPA* (Fig 2/App V). Examination of the gene revealed the presence of two introns which, when excised, yielded an open reading frame encoding 308 aa residues that aligned well with filamentous fungal lipases available in the GenBank database (Table 2/App V).

3.5.2 Transformation and expression in *T. reesei*

Suitable restriction sites were added by PCR to *lipPA* (App V) and following digestion the gene was ligated with the vector plasmid (pHEN54) and the expression construct, pHEN54lip (Fig1/App V) was successfully amplified in *E. coli*. Using biolistic bombardment of *T. reesei* conidia, 29 transformants, resistant to hygromycin B, were obtained. Using PCR with the forward primer cbh1pfwd (Table 1/App V) situated within the *cbh1* promoter region and a reverse primer, funlip2 (Table 1/App V) within *lip*PA, in combination with the hygromycin B resistance, indicated that 17 of the transformants successfully integrated the whole of the 5.5 kb cassette into the genome (Fig 3.12; Fig 1/App V). When integration occurs, it can do so at the *cbh1* locus targeted in this work or

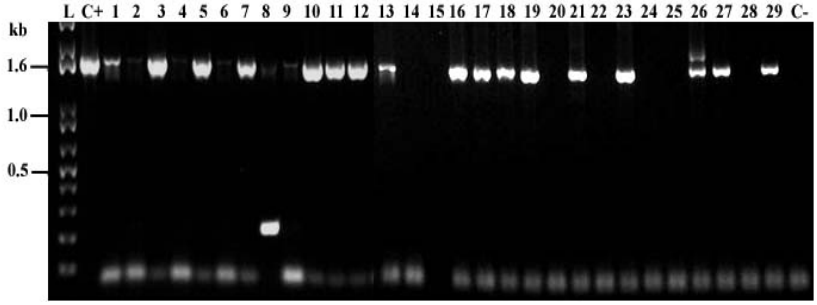

Figure 3.12 **PCR amplification products identifying successful integration of the complete pHEN54lip functional cassette into the *T. reesei* genome based on the presence of a 1538 kb PCR product**

PCR primers: cbh1pfwd and funlip2 (refer App V)
L, 1 kb Plus ladder (BRL); C+, pHEN54lip;
Lanes 1 – 29, transformants;
C-, non-transformed *T. reesei*.

at other random sites within the genome. Using the same forward primer as before (Fig 3.12) with a reverse primer designed to anneal at the extreme 3'-end of the known *cbh1* terminator region but not present on the integration cassette (Fig 1/App V), a single transformant, T16, was found to produce a PCR product of the correct size indicating integration at the desired locus. The 17 transformants and untransformed *T. reesei* were grown in liquid a culture formulated to induce lipase production under the *cbh1* promoter.

When examined by SDS-PAGE it was found that transformant T16 and some other transformants displayed a decrease of about 50% in the total secreted protein in the culture supernatant (Table 3.5), which is mainly attributable to the main cellobiohydrolase I protein (CBHI) (Fig 3.13). This reduction in CBHI is further indirect

Table 3.5 Protein concentration of supernatant of transformed and untransformed *T. reesei*

Sample	Protein concentration mg ml^{-1}
Untransformed	10.3
T1	10.3
T3	3.6
T5	10.9
T7	10.6
T10	5.6
T11	9.5
T12	5.7
T16	5.2
T17	9.9
T18	10.4
T21	8.0
T23	8.7
T29	8.5

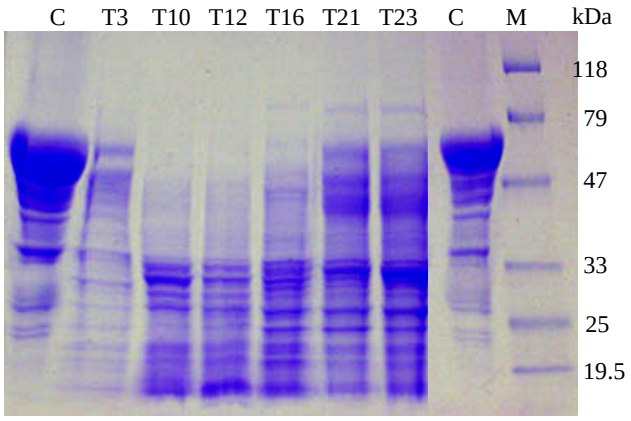

Figure 3.13 Gradient SDS-PAGE (4-20%) showing untransformed and transformed *T.reesei*

When each lane of the gel was loaded with an equal volume of supernatant, the CBHI activity bands (around 60 kDa) present in the untransformed *T. reesei* (labelled C) are significantly reduced or nearly absent in the transformants (T3 – T23). The mature LipPA protein is estimated to be approximately 31 kDa. Gel stained with Coomassie Blue R250.

evidence of integration at the targeted locus even though not supported by the PCR result for transformants other than T16.

3.5.3 Characterisation of the recombinant lipase

As the PCR and SDS-PAGE evidence (refer 3.5.2 above) indicated that T16 at least had the expression cassette integrated into the *cbh1* locus, further assays of the recombinant lipase were performed with this transformant.

A renatured SDS-PAGE gel was assayed for lipase activity with α-naphthyl acetate as substrate and Fast Blue BB Salt as indicator. This substrate is primarily suited for the assay of esterase activity, but will indicate the presence of any enzyme, including lipases, which cleave the ester bond in the naphthyl acetate (Higerd & Spizizen, 1973). Activity bands appear whenever enzymes interact with the substrate and this resulted in a large number of bands being present on the gel for both the untransformed *T. reesei* and transformant T16. Bands were most obvious in the region occupied by CBHI and there was very strong activity present in the untransformed *T. reesei* (at about 45 kDa) which was barely detectible in T16 (Fig 3.14a). This band may correspond to the *T. reesei* acetyl esterase which has a molecular weight of 45 kDa and is known to have high activity towards α-naphthyl acetate (Poutanen *et al.*, 1990). The reduced activity in the lanes containing T16 supernatant may indicate that the induction of the heterologous lipase gene has also suppressed the production of this protein.

Activity bands appeared on the lanes containing the culture supernatant from transformant T16 which were absent on the lane containing supernatant from untransformed *T. reesei* (Fig 3.14a). When subsequently restained with Coomassie Blue,

the intensity of the activity bands appeared to have been enhanced, possibly due to the retention of the dark pigmentation. The size marker indicated that the unmatched activity

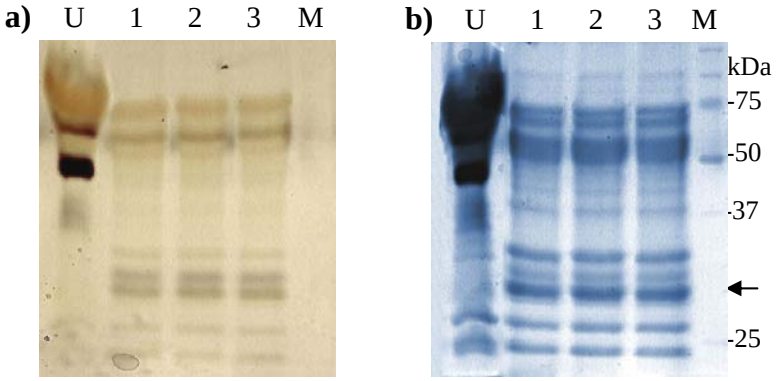

Figure 3.14 **Acrylamide gel (12.5%) showing activity bands present for the transformant but absent in the supernatant of the untransformed *T. reesei***
Renatured SDS-PAGE gel stained with **a)** α-naphthyl acetate and Fast Blue BB Salt and **b)** Coomassie Blue. U, untransformed *T. reesei*; 1-3, transformant T16; M, size marker. The arrowhead marks the approximate position of 31 kDa. The same mass of protein was loaded into each lane (50 µg).

bands were in the vicinity of 31 kDa, the approximate size of the predicted recombinant lipase (Fig 3.14b). The presence of these bands indicated either that additional enzymes or differently processed forms of a single enzyme have been secreted by the transformant which were capable of cleaving the ester bond in the α-naphthyl acetate. As the only difference between the two specimens was the introduction of the *P. allii* lipase gene, the presence of the activity can best attributed to the heterologous protein (LipPA).

When the recombinant lipase was assayed in the culture supernatant (adjusted to pH 7) using *p*-nitrophenyl caprate (C_{10}) as substrate it was found to be active between 10ºC and 50ºC with optimum activity occurring at 25ºC (Fig 3A/App V). With the reaction

temperature held at room temperature it was found that activity reached its optimum level at pH 7.9 (Fig 3B/App V). The untransformed host however failed to show any activity towards the substrate.

Additional assays were performed with the supernatants using both *p*-nitrophenyl caprate (C_{10}) and *p*-nitrophenyl laurate (C_{12}) as substrates at various combinations of temperature and pH rather than at fixed values as previously. Assays were conducted at 10ºC, 23ºC (room temperature), 37ºC and at 50ºC over a pH range 3 – 10, in all combinations. The results of these assays revealed a surprising outcome. Instead of the optimum activity being confined to the temperature and pH identified above, it was found to vary with changes in temperature and pH. In Fig 3.15a supernatant was incubated for one hour with *p*-nitrophenyl caprate as substrate and in Fig 3.15b *p*-nitrophenyl laurate was the substrate but with the incubation period increased to three hours.

A separate and quite distinct maximum was identified for each of the temperature regimes (Fig 3.15). When incubated at 50ºC, optimum activity occurred at pH 4.5; at 37ºC, pH 6; at R/T, pH 7.9 as previously shown and at 10ºC, above pH 8 most likely at

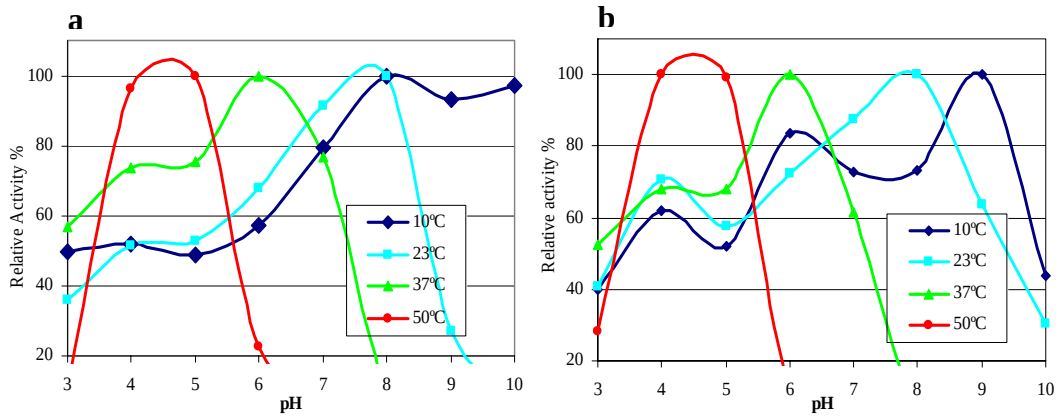

Figure 3.15 Relative activity of *LipPA* assayed at various temperatures
Activity is at the temperatures shown in the figure legend using as substrate: **a)** *p*-nitrophenyl caprate (C_{10}) incubated 1h
b) *p*-nitrophenyl laurate (C_{12}) incubated 3h.

pH 9 as seen with the longer incubation (Fig 3.15b) with the C_{12} substrate. It is also worth noting that with the longer incubations, minor peaks occurred corresponding to the maxima for higher temperatures. The untransformed *T. reesei* culture supernatant displayed none of the activity identified for LipPA from T16. Based on the actual absorbance readings obtained for the *p*-nitrophenyl released during the one hour incubation of the supernatant with the caprate, activity at 10ºC was approximately 40% and at R/T about 80% of that obtained at both 37ºC and 50 ºC.

There are no other examples in the literature describing similar variations in the activity pattern of other lipases or other members of the α/β hydrolase superfamily. It has not been established if this is a phenomenon limited to this particular lipase, LipPA, or is merely evidence of a more general action common among this class of protein.

A possible explanation for this manifestation lies in the very ubiquitous nature of lipases. In order for lipases to function in the natural world, they must operate across a broad pH spectrum, from highly acidic environments to those which are extremely alkaline. Conventionally, the action of lipases requires the three amino acids of the catalytic triad, aspartate, histidine and serine, to act collectively to cleave the ester bonds of the triglycerides (Herggård *et al.*, 2000). The picture presented here would seem to indicate that these amino acids are progressively protonated as their environment becomes increasingly acidic and act individually rather than in concert; at high pH (8 - 9), the serine being active agent, histidine around pH 6-7 and the aspartate as pH falls to 4 - 5. The presence of the multiple activity peaks which are clearly seen in Figure 3.15b may reflect changes in the proteins' tertiary structure which can arise as residues re-orientate with changes in temperature and pH.

It has been established in this work that PCR methodology can be used to isolate a lipase gene directly from genomic DNA notwithstanding the low level of homology inherent in the genes that confer lipase activity. It has also been demonstrated that such a gene can be successfully integrated into a heterologous fungal host to produce a functioning protein. Although the levels of the recombinant lipase secreted into the growth medium by its heterologous *T. reesei* host were low, the assay results obtained indicated that they exceeded that which could be induced from the native species. Examination of the published profiles of commercially available lipases (Table 3.6), suggests that the recombinant LipPA has attributes beyond those currently available.

In the course of this study, a gene has been isolated from the gDNA of an Antarctic microfungal species and the enzyme it encodes was found to have novel characteristics. This outcome exemplifies the biotechnological potential of Antarctic filamentous microfungi. With its store of fungi, the Antarctic may not only yield new lipases, but many other enzymes bearing new or unusual characteristics.

3.5.4 Modelled structure of the protein encoded by *lipPA*

The closest homologue of LipPA is the triacylglycerol lipase of *Thermomyces lanuginosus* (Table 2/App V). The crystal structure of *Thermomyces lanuginosus* lipase (TlL) has been elucidated and the three-dimensional structure of the protein is available through ExPASy (PDP_1TIB.pdb). Using the structure as a template, a 3-D model of the likely mature recombinant protein was generated which allowed a comparison to be made between the features of the two proteins. The overall structure of the proteins can be seen in Fig 3.16 showing the common arrangement of their secondary structures. The active site triad of the *Thermomyces lanuginosus* lipase (TlL): S146, D201 and H258, and oxyanion hole formed around R81 are indicated on Fig 3.16 (A1), and the corresponding

Table 3.6 Characteristics of Industrial Lipases (From Godfrey & West, 1996)

Company	Product Name	Source	pH Opt./ Range	Temp °C Opt. /range
Amano Pharmaceutical Co.	Lipase A	*Aspergillus niger*	6.5	45
	Lipase AY	*Candida rugosa*	7	40
	Lipase F	*Rhizopus oryzae*	7	40
	Lipase G	*Penicillium camembertii*	5	45
	Lipase M	*Mucor javanicus*	7	37
	Lipase **N**	*Rhizopus niveus*	7	45
	Lipase PS	*Pseudomonas fluorescens*	7	50
	Lipase R	*Penicillium roquefortii*	7	30
American Laboratories	Lipase	Porcine	7 – 8	37
	Pancreatin	Porcine	7 – 8	40
Biocatalysts Ltd	Lipomod 29P	Porcine/fungal mix	5 – 8	40 – 50
	Lipomod 34P	*Candida cylindracea*	5 – 8	45 – 55
	Lipomod 41P	Animal/fungal mix	7 – 9	35 – 45
	Lipase 50P	*Chromobacterium viscosum*	5 – 9	50 – 60
	Lipase 54P	*Mucor miehei*	7 – 8	45
	Lipase 56P	*Pseudomonas fluorescens*	7 – 8	45
	Lipase 115P	Porcine pancreas	6 – 9	45 – 55
	Lipase 187P	Fungal	5 – 7	40 – 50
	Lipase 224P	Pancreatic	7.5	30 – 45
	Lipase 299P	Pancreatic	7.0	35 – 45
	Lipase 309P	Animal/fungal mix	7.5	40 – 50
	Lipase 338P	*Penicillium roquefortii*	5 – 7	40 – 50
Enzyme Development Corporation	Enzeco Esterase	Lipase *Mucor miehei*	6 – 7	40 – 50
	Enzeco Lipase Conc	*Candida cylindracea*	6 – 7	30 – 40
	Enzeco Lipase XX	*Candida rugosa*	6 – 7	30 – 40
	Enzeco Microbial Lipase	*Aspergillus niger*	5 – 7	45 – 55

continues

Table 3.6 *continued*

Gist-Brocades NV	Piccantase	*Mucor miehei*	7.5	45 – 50
Meito Sangyo Co	Lipase MY	*Candida cylindracae*	6 – 7	40 – 50
	Lipase OF	*Candida cylindracae*	6 – 7	40 – 50
	Lipase PL	*Alcaligenes* spp.	7 – 8	50
	Lipase QLM	*Alcaligenes* spp.	7 – 9	65 - 70
	Lipase AL	*Achromobacter* spp.	10	45
	Lipase SL	*Pseudomonas cepacia*	7 – 9	65 – 70
	Lipase TL	*Pseudomonas stutzeri*	7 – 8	50
	Lipase UL	*Rhizopus* sp	6.5 – 7	40 – 45
Nagase Biochemicals	Lipase	*Rhizopus japonicus*	7	40
Novo Nordisk AS	Greasex	Lipase ns	8 – 10	ns
	Lipolase	*Thermomyces lanuginosus**	8 – 11	30 – 40
	Lipozyme	*Mucor miehei*	6 – 8	30 – 50
	Palatase M	*Rhizomucor miehei*	5 – 7	30 – 50
	Resinase 2X	Lipase ns	5 – 8	50 – 70

*GMO - expressed in *Aspergillus oryzae*
ns = not stated

residues for the modelled recombinant protein (LipPA): S143, D199, H261 and R78, are shown on Fig 3.16 (B1). The positioning and similarity between these elements in the two proteins can be clearly observed adding further support for the imputation that LipPA is a lipase.

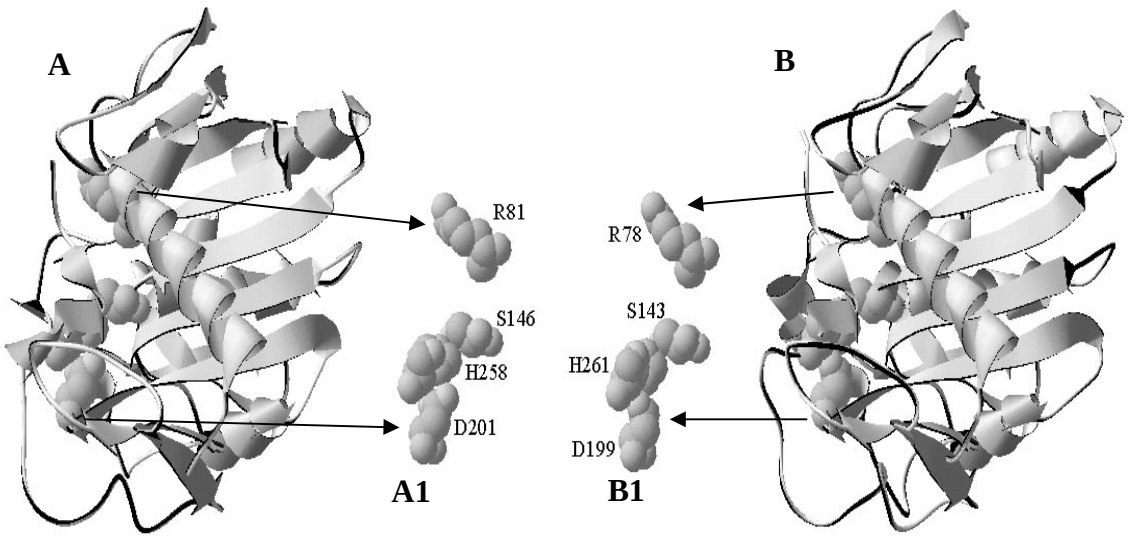

Figure 3.16 Schematic representation of (A) TlL and (B) LipPA demonstrating the close correspondence of their secondary structures

A1 and B1 illustrate the position and orientation within the models of the respective active site triads and the conserved arginine in the oxyanion holes. This figure was generated with DeepView - The Swiss-PdbViewer
Where R = arginine, S = serine, H = histidine and D = asparagine.

Although the modelling programs have a tendency to squeeze a protein to fit the model template, the Ramachandran plot for LipPA (Fig 3.17B) shows that all but a few of the residues fall within the favoured and allowed regions (Lovell *et al.*, 2003).

In a polypeptide the main chain N-Cα and Cα – C bonds are relatively free to rotate and these rotations are represented by the torsion angles Φ and Ψ, respectively. The Ramachandran diagram plots Φ versus Ψ backbone conformational angles for each residue in a protein. Statistical analysis of the structure of known proteins has identified the most favourable low-energy regions and those where there is minimal steric hindrance. The Ramachandran plot provides a means of evaluating a structure that has

been applied to a protein model. As noted by Lovell *et al.* (2003), genuinely strained conformations are often useful indicators of biological significance.

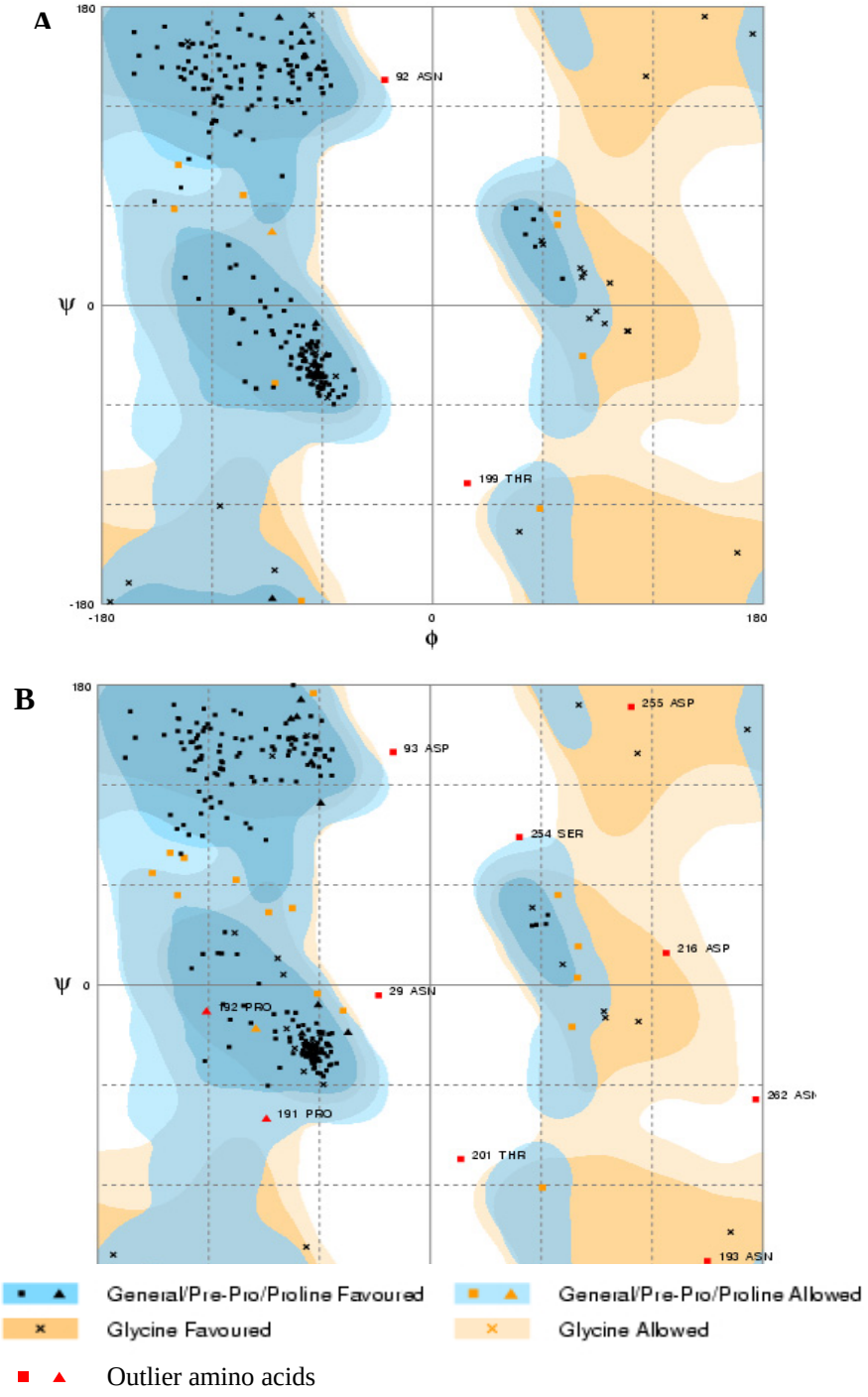

Figure 3.17 Ramachandran plots generated for (A) TlL and (B) LipPA.
These plots were generated by RAMPAGE - *Ramachandran Plot Analysis*, available through Department of Biochemistry, University of Cambridge http://raven.bioc.cam.ac.uk/rampage.php

An examination of the plots for the template, TlL, and for LipPA (Fig 3.17) indicates that two of the outliers are common. Of the remaining eight outliers which appear in Fig 3.17B, the two proline (PRO) and the serine (SER) residues lie only fractionally outside of their permitted regions and the remainder are all either asparigine (ASN) or aspartic acid (ASP). Gianese *et al.* (2001) observed in their comparative analysis of psychrophilic enzymes with their mesophilic/thermophilic analogues that asparagine is a thermolabile residue and its frequency was found to be increased in psychrophilic enzymes. When the position of the outlier residues was located on the modelled protein (not shown), it was observed that each amino acid was located on an exposed surface and generally on a random coil (the exception being Pro191/Pro192/Asn193 which lie at the end of a ß-strand).

The results for the Ramachandran plots together with the above observations would seem to reinforce the contention that the model generated represents a close approximation to the three-dimensional structure of the mature recombinant protein.

4. Conclusions and Future Prospects

This study of Antarctic microfungi would seem to have merely scratched the surface when considering the continent as a potential bioresource. From two widely separated regions of the continent, microfungi have been isolated and identified using molecular techniques. Many of the fungal genera identified have not previously been recognised in the Antarctic and the ITS sequence data of some have failed to find any matches in the records available in the public databases. The minor variations in the nucleotide sequences that were identified in similar isolates, recognised for convenience in this work as belonging to the same species, may in fact indicate an even greater wealth of diversity if not in actual speciation then at least as a multiplicity of strains.

To date the number of genera and species of microfungi recognised as emanating from the Antarctic is severely limited. The identification of this Antarctic fungal material has largely been based on morphological characteristics. It has been demonstrated here that this method of classification is open to error, particularly when directed at the all too common dematiaceous hyphomycetes, suggesting that the record of 'known' Antarctic microfungal species may represent a significant understatement.

In a recent survey of oil contaminated sites in the Ross Sea region (Aislabie *et al.*, 2001) it was found that elevated numbers of culturable microfungi were present in the contaminated soils. Some 100 isolates from at least 9 genera (identified morphologically) were recovered during the course of this survey (personal communication, Dr. J. Aislabie). Not only does this survey again point to the wealth of microfungal material present in the Antarctic but also indicates the presence of strains able to secrete cold-adapted enzymes capable of aiding in the degradation of hydrocarbons or their metabolites.

Turning to the survey of hydrolytic enzymes secreted by the Antarctic isolates, it was seen that these organisms exhibited a capacity to hydrolyse a variety of substrates over a range of temperatures. The ability of these isolates to sustain relatively high levels of enzymatic activity over a broad range of temperatures, particularly in the lower range (20º - 40ºC) as shown in the hemicellulase assays, is indicative of the potential of the Antarctic to yield microfungi bearing new and novel enzymes. As there is a clear demand by industry to acquire efficient cold-adapted enzymes, Antarctic microfungi have demonstrated their potential to fulfil this need, at least as a source for the genes encoding them.

The investigation into the physiology of the microfungi suggested that trehalose did not play a significant role as a cryoprotectant nor did glycerol contribute as a compatible solute for protection against osmotic stress. This finding that these compounds do not fulfil their ascribed roles suggests that some other compounds must be present that do offer protection against the environmental extremes. Knowledge and acquisition of these compounds could well constitute valuable assets which may have far reaching implications, particularly for cryoprotectants.

Although the investigation into ability of Antarctic microfungi to obtain protection against the threat of elevated ROS levels brought about by oxidative stress were inconclusive, the range of techniques available for the study of filamentous fungi has been expanded through the application of flow cytometry. The flow cytometer has the capacity to become a powerful tool in biotechnological research with microfungi. Due largely to its ability to examine individually the characteristics of very large numbers of germinating spores within a very short timeframe (million plus spores/specimens per hour) it has the potential to significantly improve the chances of identifying fungal

mutants and transformants on a much shorter timeframe than can be accomplished with current plating methods. Considerable further work needs to be undertaken with this device to establish its full potential.

The isolation of a lipase gene from the genomic DNA of an Antarctic *Penicillium* and the expression of a functioning enzyme by a heterologous mesophilic host has wide implications for Antarctic microfungal research. Although a microfungus may show an indication of the presence of a novel enzyme when screened for example on an indicative plate, its ability to produce the enzyme in useful quantities may be limited. The ability to isolate a gene of interest directly from genomic DNA using PCR techniques provides an avenue for prospecting the Antarctic microfungi without the need for extensive culturing or the complication of genomic or cDNA libraries.

Not only did the lipase gene isolated from *P. allii* and expressed in *Trichoderma reesei* yield a protein having novel cold-adapted characteristics but also displayed a profile unlike that seen in conventional lipases. The presence of multiple activity maxima which appear dependent on pH and temperature, pH 4.5 at 50ºC, pH 6 at 37ºC and pH 8 at room temperature and pH 9 at 10ºC, call for further investigation. The phenomenon may be a general characteristic of lipases previously unidentified or it may be unique to this protein or common among lipases from Antarctic sources. Further investigation is needed to provide an answer.

This work has contributed considerably to expanding our knowledge of Antarctic microfungi and identifying their potential as a bioresource. The study has shown that there is a wealth of microfungal material available in the Antarctic microbiota and work in this thesis has demonstrated the potential of these psychrophilic and psychrotolerent

fungi to yield genes responsible for the production of novel cold-adapted enzymes and proteins which may prove to be of considerable value to industry.

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Short Communication

Qualitative assessment of hydrolytic activities in antarctic microfungi grown at different temperatures on solid media

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Summary

Microfungi from Antarctica were grown at 10 °C, 21 °C, 28 °C and 37 °C on a series of plates each containing a single carbon source and designed to indicate the secretion of particular hydrolytic enzymes. Colony radius and hydrolytic activity were measured and a relative activity index (RA) established. In general, effective hydrolysis occurred at mesophilic temperatures. Some enzymes, especially of *Trichoderma* spp. and *Penicillium* spp. showed maximum activity at 10 °C, indicating adaptation to the colder temperatures of the antarctic environment.

Introduction

Over 150 fungal species have been described from continental Antarctica (reviewed in Onofri *et al.* 1992), but very little is known about their enzyme activities. Specific fungal enzymes may contribute to the ability to grow and survive in the harsh environment characterized by low temperatures and limited availability of organic matter. As a first step towards characterization of enzymes in antarctic microfungi we report the qualitative profiling of hydrolase activities of seven antarctic fungal isolates at different growth temperatures.

Materials and Methods

Fungi isolated from ornithogenic soils in the Windmill Islands, continental Antarctica (lat 66°17′S long 110°32′E) were provided by Dr. R.D. Seppelt (Australian Antarctic Division, Kingston, Tasmania). These included three Penicillia, nominated as *Penicillium* lake isolate, *Penicillium* sp. 2 and *Penicillium* sp. 17, *Alternaria alternata*, *Phoma* sp. and two *Trichoderma* isolates sp. 10 and sp. 10-b. Hydrolase activities were screened on minimal medium plates (Nevalainen & Palva, 1978) containing the substrate for each particular hydrolytic activity. Substrates were birchwood xylan (0.5% w/v) for xylanase, Azurine cross-linked pachyman (0.1% w/v) for β-glucanase, glucose (2% w/v) plus skim milk (1% w/v) for protease (Mäntylä *et al.* 1994), olive oil (2.5% w/v) plus rhodamine B (0.001% w/v) for lipase (Kouker & Jaeger 1987), Walseth cellulose (0.5% w/v) for cellulase (Walseth 1952), locust bean gum (0.5% w/v) for mannanase (Rättö & Poutanen 1988; Teather & Wood 1982) and chitin (0.3% w/v) for chitinase (O'Brien & Colwell 1967). Plates containing 35 ml of growth medium were used throughout the work.

For each isolate, four replicates were prepared. Plates were incubated at 10 °C, 21 °C, 28 °C and 37 °C for seven days. Hydrolase activity was visualized by the formation of a clearing or fluorescing zone around the fungal colony. For each of the four replicates, the diameter of each colony and activity zone was measured in two dimensions at 90° to each other and the values averaged. Individual colony/activity diameters differed from the overall average by no more than 5%. An index of relative enzyme activity (RA) for each isolate, substrate and temperature combination was calculated by dividing the total area of activity (the area of the clearing zone less the area of the colony) by area of the colony.

Results and Discussion

A gross measure of the production of enzymes for the degradation of a given substrate is provided by the index of relative activity (RA). In Figure 1(A–D) relative activity is illustrated for selected substrates for each fungal species and growth temperature. Proteases and hemicellulases (xylanase and β-mannanase), were most

effectively secreted into the growth environment by all isolates at all the different cultivation temperatures (Fig. 1A, B, C). The high RA values for the hemicellulases exhibited by the *A. alternata* at 37 °C (Fig. 1B, C) emphasizes the potential of this species to respond to higher growth temperatures. In the low temperature range, chitinase production by the *Penicillium* and *Trichoderma* isolates was effective at 10 °C (Fig. 1D) suggesting true adaptation to chitin degradation in the cold. In *Penicillium* sp. 2 and the *Trichoderma* isolates, strong chitinolytic activity both at 10 °C and 28 °C but not at the intervening temperature (Fig. 1D) suggests that different sets of enzymes are synthesized at different temperatures. Interestingly, a relatively high amount of active acid protease was secreted by *Trichoderma* sp. 10-b at 10 °C (Fig. 1A). Production of lipase was highest at moderate temperatures in all strains studied. The *Trichoderma* isolates grew very aggressively on this substrate (not shown). In summary, the antarctic isolates of microfungi studied showed general growth characteristics and hydrolase production resembling those of mesophilic species. However, some strains and enzymes showed adaptation to elevated and/or colder temperatures indicating the development of means to deal with environmental stress generated by non-mesophilic growth temperatures. The relatively good growth and chitinase activity displayed by *Penicillium* and *Trichoderma* isolates at 10 °C as well as protease activity in *Trichoderma* sp. 10-b prove the potential of antarctic isolates as a source of novel cold-active enzymes.

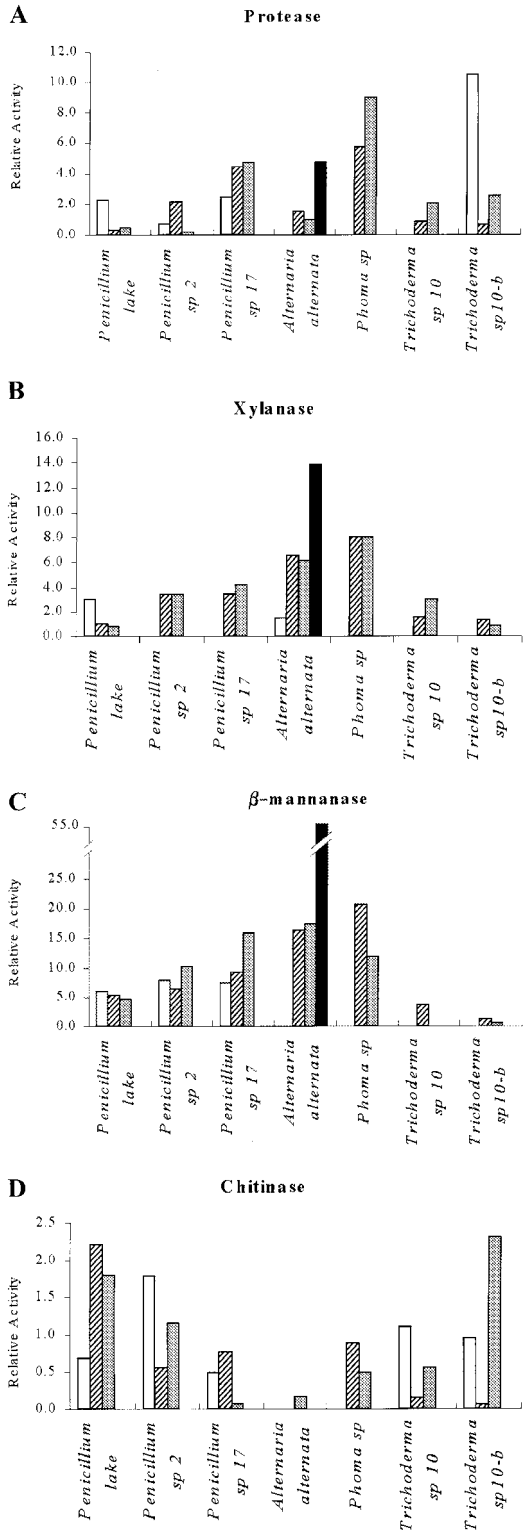

Figure 1. Relative enzyme activity displayed by Antarctic microfungi incubated at different temperatures on selected substrates. A: 2% glucose, 1% skim milk; B: 0.5% birchwood xylan; C: 0.5% locust bean gum; D: 0.3% chitin. Relative activity was calculated from the averaged values of four replicates as described in Materials and Methods. Maximum difference between measurements was 5%. Incubation temperature: □ 10 °C; ▨ 21 °C; ▩ 28 °C; ■ 37 °C.

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Hemicellulase activity of antarctic microfungi

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J.R. BRADNER, R.K. SIDHU, M. GILLINGS AND K.M.H. NEVALAINEN. 1999. The mannanase (endo-β-1,4-mannanase; E.C. 3.2.1.78) and xylanase (endo-β-1,4-xylanase; E.C. 3.2.1.8) activity of five microfungal isolates from Antarctica were characterized at different temperatures and pH. In general, the hemicellulase activity of the antarctic strains occurred at least 10 °C and as much as 30 °C lower than that of a mesophilic reference strain. At 0 °C, two strains, a *Phoma* and a *Penicillium*, produced in excess of 40% of their measured maximum activity of mannanase. All strains had maximum hemicellulase activity in the range pH 4–5, with *Penicillium*, *Phoma* and *Alternaria* strains exhibiting high (in excess of 80% of maximum) mannanase activity at pH 10. Three of the antarctic isolates exhibited high levels of xylanase activity over a pH range of 3–11.

INTRODUCTION

Filamentous fungi are found in all climatic zones ranging from the poles to the tropics. Previous reports describe over 150 fungal species isolated from soil and water samples collected in continental Antarctica (reviewed by Onofri *et al.* 1992). The focus of these studies has mainly been on the distribution and identification of fungi isolated from different antarctic environments varying in salinity, humidity and nutrient content (Ugolini and Starkey 1966; Sugiyama *et al.* 1967; Toyoda *et al.* 1985; Kerry 1990; Azmi and Seppelt 1998). To date, very little is known about the enzyme activities of antarctic filamentous fungi. As natural scavengers, fungi can use a wide range of compounds as carbon and nitrogen sources and secrete a variety of enzymes, such as cellulases and hemicellulases, to break down recalcitrant plant polymers into simple sugars for energy and growth. In our earlier work (Bradner *et al.* 1999), a qualitative investigation was made of a range of microfungi, isolated from the antarctic, to ascertain their ability to grow on solid media containing only single carbon sources. In that investigation it was found that relatively high levels of hemicellulase (mannanase and xylanase) activity could be detected over a range of growth temperatures (10–37 °C) for a number of the fungal species grown on locust bean gum and birchwood xylan.

So far, the majority of hemicellulase preparations used in industry originate from mesophilic or thermophilic organisms. These hemicellulases have found applications in a broad range of industrial processes, such as enzymatic bleaching of paper pulp, fruit-juice extraction, removal of sediments in the manufacture of instant coffee and in the bread and animal feed industries (reviewed by Godfrey and West 1996). Hemicellulases active at low temperatures could have an advantage in the production of enzyme mixtures for improvements to juice colour and clarity and increased flow rates through the ultrafiltration equipment with concomitant ease in equipment clean-up. In addition, frozen dough products could benefit from the addition of cold active hemicellulases.

In this work we report the characterization of the pH and temperature optima of xylanase and mannanase enzymes in a range of fungal species isolated in Antarctica with a view to identifying potential sources of novel enzymes.

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MATERIALS AND METHODS

Fungal strains

Antarctic fungal isolates were provided by Dr R.D. Seppelt of the Australian Antarctic Division, from a collection obtained from Windmill Islands, continental Antarctica (lat. 66°17′ S, long. 110°32′ E). The isolates were collected from ornithogenic soils in penguin colony areas. The five Antarctic strains used in this study consisted of three penicillia, *Penicillium expansum*, *P. hirsutum* and *P. commune*, *Alternaria*

alternata and *Phoma* sp. 2. A mesophilic *Trichoderma reesei* strain VTT-D-79125, which is recognized as a high secretor of hydrolases (Bailey and Nevalainen 1981), was used as a reference strain. All strains were stored on potato dextrose agar (PDA) slants held at 4 °C.

Culture conditions

Cultivation was carried out in a medium containing (g l^{-1}): Avicel, 30; soy bean flour, 15; KH_2PO_4, 15; $(NH_4)_2SO_4$, 5; $MgSO_4.7H_20$, 0·6; $CaCl_2.2H_2O$, 0·6; Tween 80, 200 μl. Fresh conidia from each strain were washed from the PDA slant with 2 ml saline solution (0·9% w/v) containing Tween 80 (0·01% v/v) into 250-ml Erlenmeyer flasks containing 50 ml of growth medium and cultured for 7 d at 25 °C on an orbital shaker set at 200 rev min^{-1}. Enzyme activities were determined from culture supernatant fluids that had been freed of fungal mycelia and remnants of the growth medium by centrifugation at 2000 *g* for 30 min and then at 10 000 *g* for a further 5 min.

Enzyme assay

Substrates. The substrate to assay for mannanase activity and to determine the temperature profile was prepared by homogenizing (using a kitchen blender) 1·0 g of locust bean gum in approximately 160 ml 0·05 mol l^{-1} Na-citrate buffer (pH 5·3) at 60 °C. The homogenate was heated to boiling point with continuous stirring and allowed to cool. The solution was covered and slowly stirred overnight at 4 °C and then made up to 200 ml with Na-citrate buffer. The method of Bailey *et al*. (1992) was used to prepare the substrate to assay xylanase activity and to determine the temperature profile. For the pH profiles, substrates were prepared by adding 1% (w/v) birchwood xylan and 0·5% (w/v) locust bean gum to 50 ml of the appropriate buffer solution and microwaving on high power for approximately 30 s until fully dissolved. All substrates were centrifuged at 2000 *g* for 10 min to remove any undissolved material and stored in 15-ml aliquots at −20 °C until ready for use.

Activity. The method of Bailey *et al*. (1992) was used to quantify the level of enzyme activity present in the culture supernatant fluid. To determine the relative activity of mannanase and xylanase enzymes present in the supernatant fluid, the quantities of the reagents in the above method were modified as follows: substrate (480 μl) and enzyme supernatant fluid (320 μl) were incubated for 10 min at the appropriate temperature and the reaction terminated by the addition of 1·2 ml dinitrosalicylic acid. When determining pH activity, the incubation temperature was maintained at 50 °C. All assays were performed in triplicate and absorbances were measured at 540 nm using a Beckman DU640 Spectrophotometer (Beckman Instruments Inc., Fullerton, CA, USA). Experiments were selectively repeated to confirm results.

Buffers. For temperature profiles and enzyme quantification, 0·05 mol l^{-1} Na-citrate buffer, pH 5·3, was used. For pH profiles, 0·120 mol l^{-1} Universal Buffer (Britton and Robinson 1931) was used for all xylanase assays and also for mannanase determinations in the pH range 2–6. Mannanase activity at higher pH values was assayed as follows: pH 7–8, 1,3-bis[tris (hydroxymethyl)methylamino]propane (bis-tris propane); pH 9, 2-[N-cyclohexylamino]ethanesulphonic acid (CHES); pH 10–11, 3-[cyclohexylamino]-1-propanesulphonic acid (CAPS), all at 0·05 mol l^{-1}.

Protein assay

Soluble protein was assayed with the Bio-Rad DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA) using bovine serum albumin as standard. The concentration is expressed in mg ml^{-1}.

Chemicals

Avicel PH-101 was obtained from Fluka Chemika (Buchs, Switzerland). Soybean flour (S-9633), birchwood xylan (X-0502), locust bean gum (G-0753), bis-tris propane (B-9410), CHES (C-2885) and CAPS (C-6070) were obtained from Sigma Chemical Co., St Louis, MO, USA. All other chemicals were obtained from BDH Chemicals Ltd, Poole, England.

RESULTS

The amount of soluble protein and the actual levels of enzyme activity measured for the culture supernatant fluids are shown in Table 1. In view of the very low values determined for the

Table 1 Protein amount and enzyme activity present in the culture supernatant fluids of fungi

Fungal isolate	Protein (mg ml^{-1})	Enzyme activity (nkat ml^{-1})	
		Mannanase	Xylanase
Penicillium expansum	0·63	1·5	3·6
P. hirsutum	0·59	16·7	8·8
P. commune	1·38	2·7	4·4
Alternaria alternata	0·45	3·2	3·3
Phoma sp. 2	0·67	8·3	3·3
Trichoderma reesei	8·59	499	1436

enzyme activity, the modified method, described in Materials and Methods, was applied in subsequent assays in order to amplify the absorbance levels when activity was measured with the spectrophotometer. For each enzyme profile, the highest measured value of activity was defined as having a relative activity of 100%. Each of the other measured values was then expressed as a percentage of this value.

Mannanase activity

Temperature profile. For the antarctic fungal strains, mannanase activity was measured at 10 °C intervals between 0 and 80 °C and extended to 90 °C for the reference strain, *T. reesei* (Fig. 1). The level of activity for each of the antarctic strains reached a maximum between 40 and 60 °C (Fig. 1), whereas maximum activity for the mesophilic reference strain occurred in the region of 70 °C (Fig. 1b). It is also notable that each of the strains under investigation (with the possible exception of *A. alternata*) exhibited a high level of activity over a broad temperature range, with levels of activity in excess of 50% of the maximum over a 40–65 °C range (Fig. 1). This is clearly different to the activity levels measured for *T. reesei*, which indicated activity in excess of 50% of the maximum over a range of less than 30 °C (Fig. 1b).

A comparison of the profile presented here for the reference strain with that of each of the antarctic isolates (Fig. 1) indicated the consistently higher levels of activity present at low temperatures in the latter group. Of particular note are the *P. expansum* (Fig. 1a) and *Phoma* sp. 2 (Fig. 1b) strains, both of which appeared to maintain a significant level of activity when assayed at 0 °C (49·4 and 41·7%, respectively).

pH profile. The maximum level of mannanase activity was found to lie in the range pH 3–4 for the five antarctic isolates and the reference strain (Fig. 2). Beyond this maximum, the

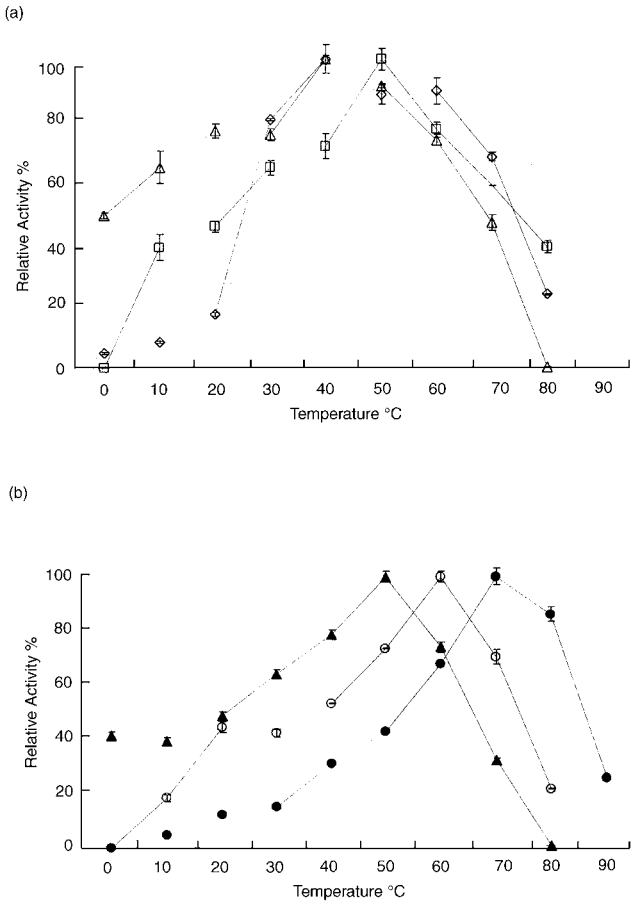

Fig. 1 Mannanase temperature profile measured using locust bean gum as substrate. (a) *Penicillium expansum* (△), *P. hirsutum* (◇), *P. commune* (□) and (b) *Alternaria alternata* (○), *Phoma* sp. 2 (▲), *Trichoderma reesei* (●). Error bars indicate S.E. of three assay replicates

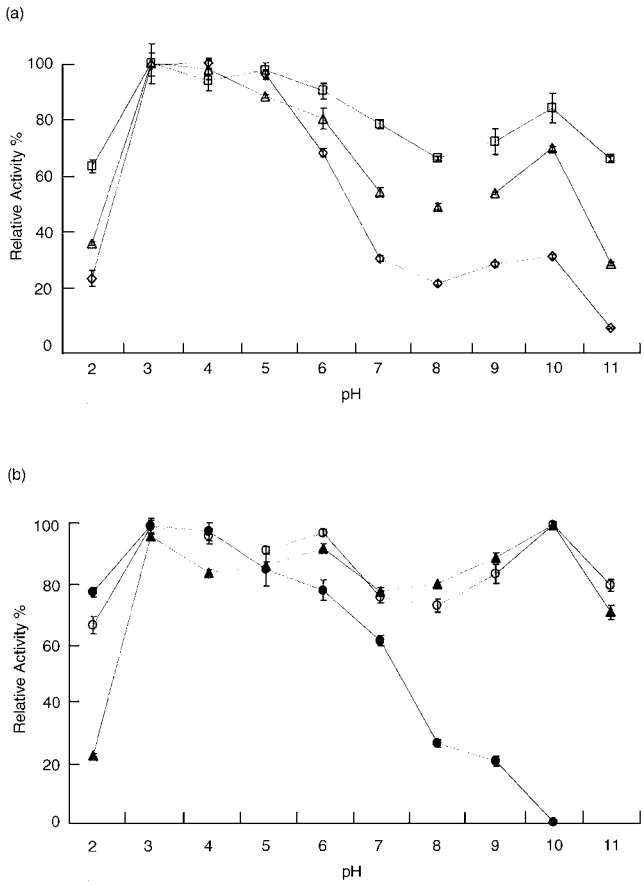

Fig. 2 Mannanase pH profile measured using locust bean gum as substrate. (a) *Penicillium expansum* (△), *P. hirsutum* (◇), *P. commune* (□) and (b) *Alternaria alternata* (○), *Phoma* sp. 2 (▲), *Trichoderma reesei* (●). Error bars indicate S.E. of three assay replicates

level of activity for *T. reesei* was observed to decline in a regular manner, reaching zero by pH 10 (Fig. 2b). The profile that emerged for the antarctic strains differed markedly from that of the reference strain. For three of the isolates, *P. commune* (Fig. 2a), *A. alternata* (Fig. 2b) and *Phoma* sp. 2 (Fig. 2b), activity levels remained in excess of 60% of the maximum across the total pH range assayed. Even more significantly, both *A. alternata* and *Phoma* sp. 2 were observed to have three separate and distinct maxima at pH 3, 6 and 10. These peaks in the levels of activity may indicate that these species secrete two or more distinct mannanase enzymes into their environment. Elevated levels of activity were also observed at pH 10 for both *P. commune* and *P. expansum* (Fig. 2a).

Xylanase activity

Temperature profile. As was found for mannanase, maximum xylanase activity was reached between 40 and 60 °C for each of the antarctic strains whilst the reference strain displayed its maximum activity at 60 °C (Fig. 3). Although not as pronounced as with mannanase, activity in excess of 50% of the maximum occurred over a broader temperature range than *T. reesei* (approximately 25 °C; Fig. 3b) for *P. hirsutum* (approximately 30 °C; Fig. 3a), *P. commune* (approximately 35 °C; Fig. 3a) and *Phoma* sp. 2 (approximately 40 °C; Fig. 3b). For this assay there was no significant activity identified at very low temperatures, with the exception of *P. expansum*, which displayed an increase in enzyme activity at 10 °C (Fig. 3a).

pH profile. The activity profiles displayed by the antarctic species appear very different from that obtained for the mesophile, *T. reesei* (Fig. 4). All species assayed indicated that maximum enzyme activity occurred at pH 4–5. However, unlike the reference strain, high levels of activity (in excess of 50%) appeared to persist over the whole (upper) pH range for all species under investigation except *P. commune* (Fig. 4a). A second (but lesser) peak in activity at pH 7–8 was observed in the profile for the *A. alternata* isolate (Fig. 4b).

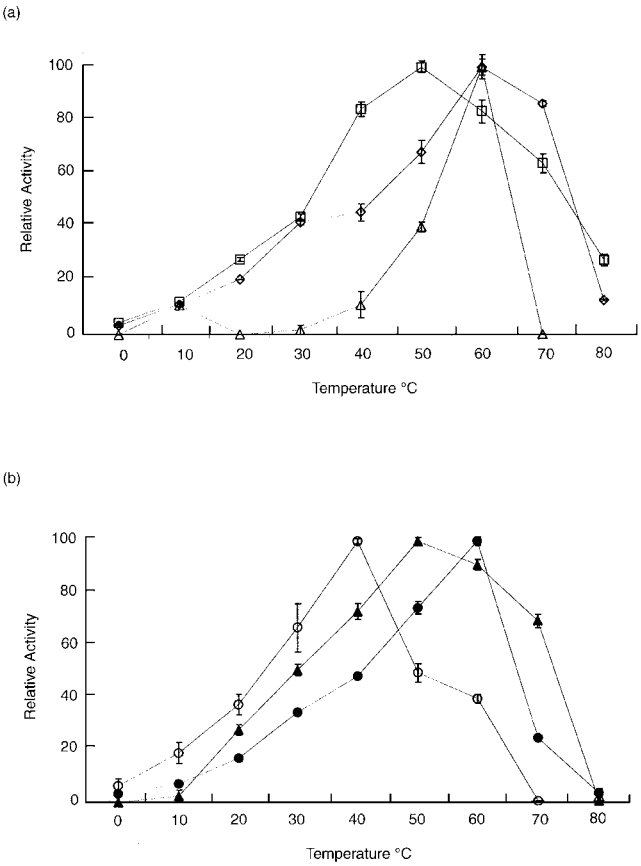

Fig. 3 Xylanase temperature profile measured using birchwood xylan as substrate. (a) *Penicillium expansum* (△), *P. hirsutum* (◇), *P. commune* (□) and (b) *Alternaria alternata* (○), *Phoma* sp. 2 (▲), *Trichoderma reesei* (●). Error bars indicate S.E. of three assay replicates

DISCUSSION

In this investigation a direct comparison has been made between the hemicellulase temperature and pH profiles displayed by the fungus *T. reesei* VTT-D-79125 and the five antarctic fungal isolates. *Trichoderma reesei* is a typical mesophilic fungus and is used extensively in the production of industrial enzymes (Godfrey and West 1996). The temperature profile for both mannanase and xylanase indicated that the antarctic isolates attained their maximum activity at a temperature up to 30 °C lower than that of the reference strain. For two strains, *P. expansum* and *Phoma* sp. 2, relatively high levels of mannanase activity were detected at 0 °C. There is a general pattern of increased hemicellulase activity by the antarctic microfungi at lower temperatures when compared with the reference strain.

The presence of high mannanase activity at both low temperatures and a wide range of pH values identified in the *Phoma* sp. 2 and *P. expansum* isolates suggests that these strains may prove to be a useful source of enzymes, particularly for food-processing, such as fruit-juice extraction. Cold acting hemicellulases may have a broader application to other industrial processes where there is a need to maintain a cold temperature environment to ensure flavour retention, to avoid denaturing essential elements of the product or simply where the addition of heat to a process is counterproductive for purely economic reasons.

Maximum hemicellulase activity of the reference strain, *T. reesei*, was found to occur at pH 4 and all antarctic isolates were also found to have a level of activity at or near maximum at this value. Of particular interest is the broad pH range

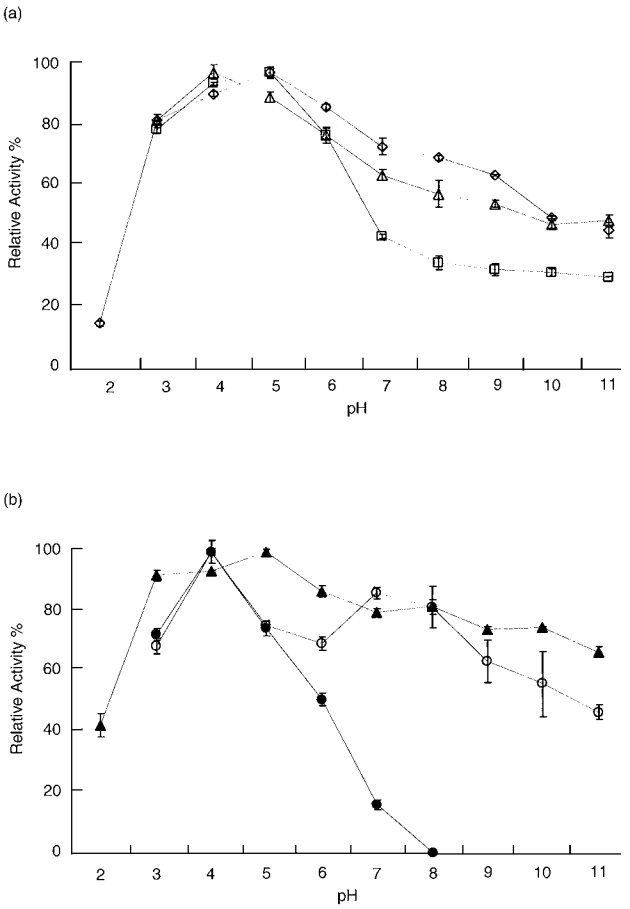

Fig. 4 Xylanase pH profile measured using birchwood xylan as substrate. (a) *Penicillium expansum* (△), *P. hirsutum* (◇), *P. commune* (□) and (b) *Alternaria alternata* (○), *Phoma* sp. 2 (▲), *Trichoderma reesei* (●). Error bars indicate S.E. of three assay replicates

over which the antarctic fungi show significant levels of activity. The emergence of multiple peaks in the mannanase profiles of each of the isolates and the xylanase profile for *A. alternata* suggests the possibility of multiple enzymes being produced. It was noted above that penguin colonies are located within the environs of the Windmill Islands. Soil samples from the substratum of this region, which are subject to penguin guano and general penguin influence, have been shown to have pH values in excess of 8 (Azmi and Seppelt 1998). This may explain the presence of enzymes capable of degrading hemicelluloses in a highly alkaline environment.

It is not known at present whether any or all of the isolates discussed in this work are native to Antarctica or have been carried there as elementally borne propagules or by animal, bird or human activity. However, it does appear that each of the isolates has undergone some adaptive processes in terms of the temperature and pH behaviour of their hydrolytic enzymes which have permitted them to endure in the extreme environment. The very character of these adaptations may well serve as a source of novel cold active enzymes and their genes.

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SHORT NOTE

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P. M. Selkirk · K. M. H. Nevalainen

A new microfungal isolate, *Embellisia* sp., associated with the Antarctic moss *Bryum argenteum*

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Abstract A microfungal isolate of *Embellisia* sp. (Simmons), assigned *Embellisia* sp2, not previously described from the Antarctic, has been identified by morphological means and internal transcribed spacer (ITS) region sequencing. *Embellisia* sp2 was shown to be associated with the bryophyte *Bryum argenteum*, collected from Marble Point in Southern Victoria Land, Antarctica, and grew only in samples plated from crushed moss tissue and surface-sterilized leafy stems. Two other types of microfungi, *Penicillium* sp. and *Trichoderma* sp., were cultivated from a surface rinse of the moss.

Introduction

A wide range of microscopic life forms including microalgae, fungi, bacteria, mosses and liverworts colonize Antarctica, where they form an integral part of the Antarctic ecosystem and play a significant role in the energy flow, transformation of organic matter and soil productivity (Vincent 1988; Vishniac 1993). Some 15 species of mosses have been reported from continental Antarctica (Smith 1984) and 8 species from Southern Victoria Land (Seppelt and Green 1998). To date, at least 30 fungal genera have been described from the moss samples collected from various sites in Antarctica (Greenfield and Wilson 1981; Möller and Dreyfuss 1996; Azmi and Seppelt 1998). In this study, microfungi were characterized from the moss, *Bryum argenteum*, collected from Southern Victoria Land.

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Materials and methods

Study site

Moss samples were collected in midsummer at Marble Point (77°24′S; 163°48′E), on the western side of the Ross Sea, in gently flowing water in Surko Stream, a melt stream from the Wilson Piedmont Glacier. Samples were dried and stored at ambient field temperature in Antarctica (approximately 0 °C) until transported to Australia by air, and then stored at −20 °C prior to examination.

Isolation of microfungi from the moss samples

Unbroken moss was rinsed with 5 ml 0.9% (w/v) NaCl + 0.01% (v/v) Tween 80 and 100 µl of the rinse solution was spread onto Potato Dextrose Agar (PDA) plates. The surface-rinsed moss was ground under aseptic conditions, suspended in 5 ml 0.9% NaCl + 0.01% Tween 80 and spread onto PDA plates. To study the mode of association of the microfungi with moss structures, individual leafy stems were separated from the main body and surface-sterilized by soaking in NaOCl (0.01% v/v) for 1 min. The stems were washed twice to remove NaOCl, dried by blotting on sterile filter paper and transferred to PDA plates. All plates were incubated for 1 week at 26 °C and fungal colonies were purified by restreaking.

Identification of microfungi

Samples from the surface rinse and the ground moss were stained with lactophenol cotton-blue and viewed by light microscopy. Identification of the cultures was based on recognized morphological criteria (Domsch et al. 1980). Where the morphological identification was controversial, molecular identification was attempted by sequencing the internal transcribed spacer region (ITS) of the nuclear ribosomal DNA. Specimens of *Curvularia brachyspora, C. trifolii* and *Embellisia* sp1 were obtained from the culture collection of the NSW Department of Agriculture and their DNA sequenced. Further sequence data used for phylogenetic analysis (Fig. 2) were obtained directly from the GenBank Nucleotide Database.

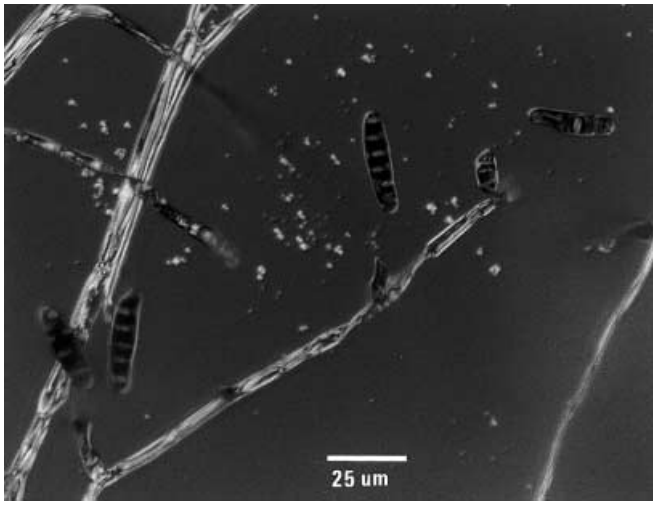

Fig. 1 Microscopic examination (×400) of the conidia of the fungal isolate associated with the Antarctic moss *Bryum argenteum* and identified as *Embellisia* sp. (Simmons)

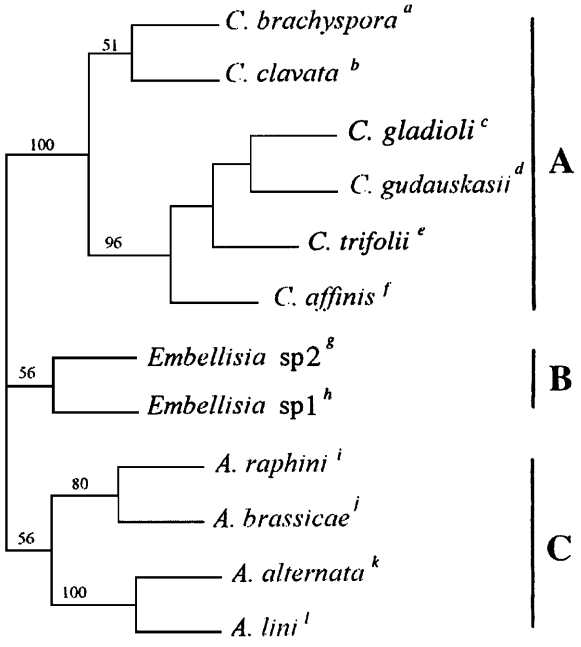

Fig. 2 Phylogenetic tree based on analysis of the ITS nrDNA sequences showing the relationship between *Curvularia* (*A*), *Embellisia* (*B*) and *Alternaria* (*C*). Numbers at branching points are percentages of 1000 bootstrapped data sets supporting the specific internal branches. GenBank Accession Numbers: *a* AF212308; *b* AF071336; *c* AF071337; *d* AF071338; *e* AF212310; *f* AF71335; *g* AF212309; *h* AF212317; *i* U05200; *j* U05253; *k* Uø5195; *l* Y17071

DNA extraction, amplification and sequencing

Fungal strains were grown on PDA plates for 5 days for the extraction of chromosomal DNA as described by Yates and Gillings (1998). The entire ITS region of nrDNA was amplified for each isolate by PCR utilizing the primers ITS 1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS 4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al. 1990). Genomic DNA was diluted 1:10 and subjected to 35 cycles of PCR using AmpliTaq Gold, with $MgCl_2$ concentration of 0.25 mM. The temperature for the annealing step was 58 °C and the reaction volume was 50 µl. The PCR product was cleaned using "QIAquick PCR Purification Kit" (QIAGEN Hilden, Germany) in accordance with the manufacturer's instructions, and sequenced directly using an ABI Prism, Model 377 automated fluorescent DNA sequencer (Perkin-Elmer). Sequences have been deposited with GenBank and the accession numbers are shown in Fig. 2.

DNA analysis

Phylogenetic analysis of the aligned sequences was performed using the PHYLIP computer package, version 3.57. Nucleotide distances were estimated with the maximum likelihood model and a tree constructed by the Fitch-Margoliash method with the complete deletion of gaps and equivocal sites. The robustness of the internal branches was tested by bootstrap analysis (Felsenstein 1985) from 1000 bootstrap replications.

Results and discussion

Two microfungi, identified as *Penicillium* sp. and *Trichoderma* sp. by both colony phenotype and conidiophore morphology, appeared in the samples from the surface rinse. Species of these genera have been shown to be frequently present in Antarctic soils (Greenfield and Wilson 1981; Azmi and Seppelt 1998). An initial inspection of the conidia of a third species (Fig. 1) tentatively classified it as a *Curvularia* sp.; however, with further detailed examination it was identified as *Embellisia* sp. Simmons (Simmons 1971), otherwise similar to *Embellisia chlamydospora* but not producing chlamydospores in culture. This isolate was assigned *Embellisia* sp2.

The ITS region of DNA extracted from the Antarctic isolate, the reference *Curvularia* strains and *Embellisia* sp1 was successfully amplified by PCR (not shown) and complete sequences were obtained. A comparison between the sequence data for the Antarctic isolate and the reference strains plus data from the GenBank Nucleotide Database revealed a considerable amount of variation in the ITS region between the isolates and within each of the genera. Figure 2 depicts a phylogenetic tree obtained by analysis of the sequence data and three distinct clusters, marked A, B and C, can be observed. Group A comprises species classified as *Curvularia*, group B as *Embellisia* and group C as *Alternaria*. The molecular analysis suggests that the Antarctic isolate has been correctly identified within the *Embellisia* group. The analysis provides strong support for classifying the *Curvularia* as monophyletic; however, the distinction between genus *Embellisia* and *Alternaria* is not as significant, which also corresponds with the findings of other researchers (Cooke et al. 1998). Notwithstanding that *Embellisia* has been classified as a genus, the evidence here suggests that this group may in fact represent a clade of *Alternaria*.

In temperate environments, members of the genus *Embellisia* have generally been isolated in association with the higher plants, sometimes as endophytes and frequently as pathogens. That *Embellisia* sp2 was not found in the surface rinse but grew only on plates

inoculated with ground moss suggests that a close association exists between the fungus and the moss. This was also implied by the growth of the same species on plates inoculated with surface-sterilized leafy stems. Microscopic examination of transverse sections of stained moss stems (not shown) did not indicate the presence of *Embellisia* sp2 in the moss tissue, ruling out an intimate endophytic relationship. The Ascomycete *Embellisia* sp2, found in this study to be closely associated with *Bryum argenteum* from Marble Point, has not been reported in earlier studies on the isolation of fungi from Antarctica, and may be sufficiently different to *E. chlamydospora* to represent a new fungal species. We believe that this is the first report on the use of ITS sequence data as a tool in the identification of an Antarctic fungal isolate.

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Metabolic activity in filamentous fungi can be analysed by flow cytometry

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Abstract

The use of flow cytometry in combination with fluorescent dyes as a technique to rapidly differentiate and enumerate bacterial and yeast cells is well established. We have shown that through the judicial choice of stains, the nondestructive screening and sorting of fungal material is possible. The early stages of growth, from germination through hyphal development of three filamentous fungal species, *Penicillium*, *Phoma* and *Trichoderma*, have been followed using forward- and side-angle scatter on a Becton Dickinson FACSCalibur flow cytometer. By staining isolates with the permeant fluorogenic substrates, dihydroethidium and hexidium iodide metabolic activity in the developing hyphae has been measured. We have been able to demonstrate that there is a 12–13 h window of opportunity during which germination and the early stages of hyphal development of filamentous fungi can be analysed by flow cytometry.

Keywords: Filamentous fungi; Flow cytometry; Metabolic activity

1. Introduction

Flow cytometry is a technique that has been developed for making rapid measurements on individual particles or cells as they flow in a fluid stream past a sensing point. The important feature of flow cytometric analysis is that measurements are made separately on each particle in turn within the suspension, and not just as an average value for the whole population.

Modern laser-based flow cytometers are capable of measuring multiple cellular parameters simultaneously based on light scatter and fluorescence. In a flow cytometer, when the laser light beam hits a stream of cells, it is scattered in all directions. This light is measured in the forward scatter or orthogonal plane and in the right angle or side scatter plane. Forward scatter (FSC) detection can be viewed as an approximation of the size of a cell or particle passing the detectors and side scatter (SSC) an approximation to granularity or shape (Shapiro, 1995). When the laser beam excites a fluorochrome, the emitted light is detected by three (sometimes more) fluorescence detectors.

A wide range of fluorescent probes are available (Haugland, 1996), which permit the direct detection of a variety of properties, such as amounts of various cell components, specific sequences of peptides and nucleotides, cell functions and enzyme activities (Bernander et al., 1998; Fitzpatrick et al., 2000; Katsuragi and

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Tani, 2000; Ormerod, 2000; Reiseberg et al., 2001; Veal et al., 2000). Some fluorochromes (fluorescent dyes) can penetrate the cell membrane and enter the cytosol whilst others can be attached to antibodies that are specific to cell surface markers or can enter the cell and interact with the cell's metabolic processes (Haugland, 1996). Natural fluorescence (autofluorescence) and fluorescent substrates can be utilized to elicit information on individual cells, cellular components and metabolic activity.

In this investigation, two fluorescent probes, dihydroethidium and hexidium, iodide have been utilized. Both stains are phenanthridinium dyes structurally similar to ethidium bromide (EtBr) and propidium iodide (PI); however, where EtBr and PI are generally unable to penetrate the cell membrane of viable cells, both dihydroethidium and hexidium iodide are cell permeant. The dye dihydroethidium (also known as hydroethidine) is a chemically reduced ethidium derivative that carries no positive charge. In its chemically reduced state, this dye exhibits blue fluorescence within the cytoplasm. In viable cells, it can be oxidized (dehydrogenated) by reactive oxygen species (ROS) to ethidium, which intercalates with DNA and fluoresces red. Hexidium iodide is a phenanthridium dye developed (and patented) by Molecular Probes (Eugene, OR, USA). By slightly increasing the size of the substituent at the 5-position of the phenanthridine ring (the ethyl group of EtBr replaced with a hexyl substituent), the lipophilicity of the probe was increased resulting in a significant improvement in the permeability of the dye through the membrane of living cells. Within the cell, this stain will exhibit orange/red fluorescence when excited by light from an argon laser (488 nm).

An important feature available on many modern flow cytometers is the ability to separate (sort) cells specifically identified during analysis (Ormerod, 2000; Vives-Rego et al., 2000). Any combination of parameters can be applied as criterion to decide whether a cell is sorted or not, and those selected can then be directed (collected) to optical slides or a suitable membrane for further analysis. Of particular importance for ongoing research is the nondestructive nature of the sort capability; organisms that are viable when introduced to the flow cytometer invariably remain viable after sorting.

The use of flow cytometry has been documented in its application to bacteria (Bernander et al., 1998; Katsuragi and Tani, 2000; Melacrinò et al., 2001; Veal et al., 2000; Vives-Rego et al., 2000), to yeast (Bell et al., 1998; Melacrinò et al., 2001) and to fungal spores (Carr and Shearer, 1998; De Lucas et al., 1998; Gourmet et al., 1997; Kim et al., 2001; Smith et al., 1999) and Hopfer et al. (2001) have reported filament formation in *Candida albicans* and have examined filamentation in heat killed *Aspergillus fumigatus.* However, other than some preliminary investigations described by Nevalainen (2001), virtually no work has been documented on the application of flow cytometry to filamentous fungi in the early stages of growth when hyphae have started to develop from germinating spores.

The aim of this work is to investigate the potential of flow cytometry as a tool for both the analysis of filamentous fungi and in strain development. In order to gain some measure of the potential of this technique, we have utilized a range of different fungal species. These included a strain of *Trichoderma reesei*, used extensively in the production of industrial enzymes; *Trichoderma viride*, an isolate known for its ability to act as a biocontrol agent; a plant pathogen *Phoma herbarum*, and lastly an isolate from a genus used in the production of antibiotics and also a potential plant pathogen, *Penicillium expansum.* The availability of an isolate of *P. expansum* sourced from Antarctica has provided an opportunity to extend the scope of the investigation. The extreme conditions which prevail in the Antarctic have the potential to cause indigenous (or introduced) species to develop adaptive metabolic strategies to meet the demands of their environment. Some of the changes such as response to high levels of UV radiation and cold-induced oxidative stress may be identified through the application of flow cytometry.

2. Material and methods

2.1. Fungal isolates and culture methods

The filamentous fungal species listed in Table 1 were used in this study. The Antarctic isolate of *P. expansum* has been identified by sequencing the internal transcribed spacer region of the ribosomal DNA and sequence data lodged with GenBank (AF218786) and samples of the isolate are held by NSW Agriculture (Plant Pathology Herbarium, Orange, NSW, Australia). All isolates were cultured for 10 days on potato dex-

Table 1
Fungal isolates used in this study

Species	Culture collection	Strain ID
Trichoderma reesei	VTT Finland[a]	VTT-D-79125
Trichoderma viride	Roal Oy Finland[b]	ALKO3092
Penicillium expansum[c]	NSW Agriculture[d]	DAR74612
Phoma herbarum	NSW Agriculture	DAR57083

[a] State Technical Research Centre, Espoo, Finland.
[b] Roal Oy, Rajamäki, Finland.
[c] Antarctic isolate.
[d] Plant Pathology Herbarium, Orange, NSW, Australia.

trose agar (Oxoid, Basingstoke, Hampshire, England) plates and fresh conidia were harvested by washing with 5 ml sterile saline solution (0.9% w/v) containing Tween 80 (0.01% v/v) and filtered through sterile cotton wool to remove any residual mycelia.

Erlenmeyer flasks (250 ml) containing 50 ml of potato dextrose broth (Difco Laboratories, Liverpool, New South Wales, Australia) were inoculated with 1 ml from each of the spore solutions. Flasks containing *T. reesei* and *P. expansum* were incubated at 28 °C on a rotary shaker at 250 rpm. Germination of the conidia was monitored by light microscopy and sample aliquots (500 μl) were removed from the cultures at commencement and after 4, 8, 9 and 10 h and submitted for staining. The whole of the experiment was duplicated for *T. reesei*. A second set of flasks was similarly inoculated with *P. expansum*, *T. viride* and *P. herbarum* but incubated at room temperature (22 °C) and shaken at 150 rpm. Aliquots were again removed and stained at the start and hourly from 9 to 13 h of incubation. Each of the experiments was replicated using conidia harvested from fresh culture plates.

2.2. Fluorescent staining

Two permeant fluorogenic substrates used in this work were obtained from Molecular Probes. Stock solutions (1 mg ml^{-1}) were prepared by dissolving dihydroethidium (DHE) and hexidium iodide (HI) in dimethyl sulfoxide (Sigma, St Louis, MO, USA). The aliquots of fungal spore/hyphal suspension were stained by addition of 1 μl of stock solution and mixed by inversion.

Cytosolic dihydroethidium when oxidized by ROS (singlet oxygen, hydroxyl radicals, superoxide, hydroperoxides and peroxides) yields ethidium, which intercalates with a cell's DNA and fluoresces bright red (605 nm). The HI stain permeates eukaryotic cells staining both the cytoplasm and nuclei and when exited fluoresces orange-red (600 nm).

2.3. Flow cytometry data acquisition and analysis

Data acquisition was performed immediately after completion of sample preparation using a FACSCalibur flow cytometer (Becton Dickinson, North Ryde, NSW, Australia) operated with Osmosol (Lab Aids, Narrabeen, NSW, Australia) sheath fluid. The excitation light (wavelength, 488 nm) was from a 15 mW argon ion laser. ImmunoCheck beads (Coulter Electronics) were analysed each day to ensure that the cytometer was correctly aligned. To keep the total data rate below 300 events per second during analysis, the flow rate was adjusted and the sample suspension was diluted with distilled water as necessary. The detection threshold in the side scatter channel (SCS) was set at a level just below the level of the lowest spore signals (at time zero). Debris (at time zero) that remained detectable in the forward scatter channel (FSC) below the level of the lowest spore signals was removed from the analysis by gating. Analysis of the data was performed using WinMDI2.8 Build #13 (Trotter, 2000) software package.

3. Results and discussion

In this study, we used forward (size) and side scatter (granularity or shape) to track the early stages of growth in different species of filamentous fungi in liquid culture and two nondestructive fluorescent stains to provide evidence of metabolic activity.

3.1. Forward and side scatter analysis

The *T. reesei* and *P. expansum* isolates were incubated at 28 °C and 250 rpm and their growth was followed for 10 h. The initial samples (time zero) were processed through the flow cytometer and the region occupied by ungerminated spores, with reference to forward and side scatter, was appropriately identified on the cytograms (Fig. 1). It was confirmed that the regions marked also encompassed all fluorescence exhibited by the spores in each of the fluorescence

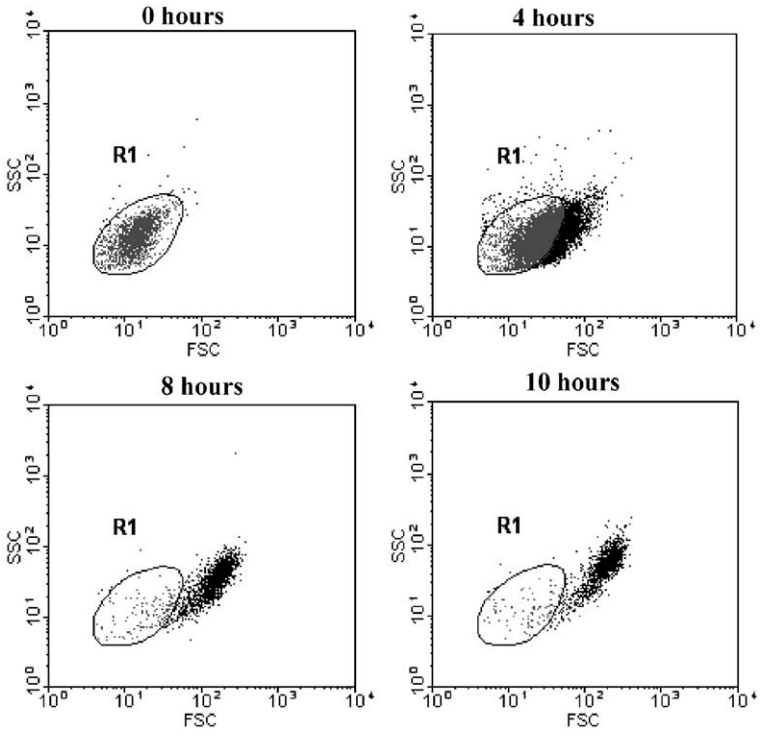

Fig. 1. Dot plots (cytograms) showing the changes in *T. reesei* spores grown in liquid culture. The region occupied by ungerminated spores has been highlighted (R1) and the relative position of the developing cohort can be clearly seen to move upwards and to the right over time. All measurements are in arbitrary units (AU).

detection channels (Table 2). After four-hour incubation, spores were observed by microscopy to have swollen (not shown), and this was reflected in the increase in the geometric mean (GM) determined in the forward scatter (FSC) channel (Table 3[i]).

At the end of eight h incubation, the development of hyphae was well advanced in both species (not shown) and this was again reflected in the ten-fold increase in the values obtained in the forward scatter channel (Table 3[i]). Presumably, reflecting the changes in shape, the side scatter values were seen to nearly triple, from 11.9 to 32.6 arbitrary units (AU) for *T. reesei*, and an increase of seven-fold from 19.0 to 146.0 AU for *P. expansum*. Over the following 2 h, the values recorded for FSC remained virtually unchanged for *P. expansum* at 260 AU but showed a steady increase, rising from 157.5 to 209.5 AU, for *T. reesei* (Table 3[i]). As noted above, development of both species was well advanced after 8 h, further growth being directed to the development of the hyphae. This proposition can be supported by following the continued development in SSC (a rough approximation to shape). The SSC values for both *T. reesei* and *P. expansum* each increased by a further 24 AU over the next 2 h (Table 3[i]). The growth and development of a cohort of *T. reesei* spores over a 10-h incubation period (measured by FSC) is clearly demonstrated in Fig. 1.

Table 2
Fluorescent detectors available in the FACSCalibur flow cytometer

Detector (channel)	Emission detection range (nm)
FL1 (green)	515–565
FL2 (orange)	565–605
FL3 (red)	>605

3.2. Fluorescence analysis

Fluorescent stains were introduced to evaluate the ability of flow cytometry to measure metabolic activ-

Table 3
Changes over time, measured by flow cytometry, in forward scatter (FSC), side scatter (SSC), HI fluorescence (FL2) and DHE fluorescence (FL3) of filamentous fungi incubated at (i) 28 °C and 250 rpm and (ii) room temperature and 150 rpm

(i)

	Trichoderma reesei					*Penicillium expansum*				
	Time (h)									
	0	4	8	9	10	0	4	8	9	10
FSC	14.1	47.0	157.5	187.4	209.5	25.6	54.1	260.6	246.8	256.9
SSC	11.9	11.4	32.6	43.6	56.5	19.0	25.7	146.0	150.8	170.3
HI	13.6	81.4	189.8	242.5	313.5	5.0	69.2	488.3	518.0	1206.0
DHE	5.4	14.7	17.1	23.5	42.4	7.4	26.2	40.6	43.5	60.1
Auto FL2	1.6	1.9	6.5	8.9	12.6	1.6	2.2	17.8	20.8	24.2
Auto FL3	2.3	2.8	7.7	9.9	14.3	2.3	3.0	18.6	21.8	28.0

(ii)

	Penicillium expansum		*Trichoderma viride*		*Phoma herbarum*	
	Time (h)					
	0	10	0	11	0	11
FSC	25.0	340.4	18.6	116.9	6.9	110.6
SSC	18.9	158.4	15.4	42.5	8.3	31.7
DHE	10.8	67.3	3.8	17.1	1.8	52.9

Measurements are shown in arbitrary units (AU). At time 0, figures represent the geometric mean (GM) of the 10,000 events recognized by the flow cytometer. At later times, values shown are the GM as the spores develop into hyphae. Nongerminating spores have been removed by gating. The level of autofluorescence displayed by each species is shown as Auto FL2 and Auto FL3.

ity in developing filamentous fungi. If hexidium iodide enters the growing fungi, it can be excited by the argon laser and emitted fluorescence detected in the orange FL2 channel (detection is also possible in the FL3 channel on the FACSCalibur). This fluorochrome was found to produce high levels of signal that was consistent with the stain interacting with both the cytosol and the nuclei of the cells (Haugland, 1996) providing yet another measure of overall growth and development of the fungi. With the *T. reesei* strain, detected fluorescence rose from a basal level (at time zero) of 13.6 AU to a value of 313.5 AU after 10-h incubation. The result obtained with *P. expansum* proved even more dramatic—the value rising from a base of 7.4 to 1206.0 AU at 10 h (Table 3[i]). These results reinforce the implications above that the growth of the *Penicillium* exceeded that of the *Trichoderma* over the timeframe of the experiment.

In using dihydroethidium as an analytical stain, we have attempted to demonstrate that flow cytometry can be utilized to recognize enzymatic activity occurring within the developing fungus. The DHE stain does not itself fluoresce when subjected to the blue laser of the FACSCalibur, but acts as a probe for the generation of ROS within the cell. Reactive oxygen species frequently arise when an organism is subjected to stress and in their presence, dihydroethidium is oxidized to ethidium which intercalates within the cell's DNA, staining its nucleus a bright fluorescent red. As free radical species can be deleterious to the fungus, being a potential source of chromosomal damage, fungi (and other eukaryotes) produce anti-oxidants (such as superoxide dismutase) as a counter-measure. The greater the ability of a fungal species to produce anti-oxidants, the lower will be the level of free radicals and hence the lower will be the amount of ethidium detectable by the flow cytometer. In this work, we followed the development of fluorescent activity over a 10-h time frame resulting from the intercalation of ethidium with the DNA of *T. reesei* and *P. expansum*. After adjusting for autofluorescence, the *Trichoderma* yielded a nine-fold increase ([42.4 – 14.3]/[5.4 – 2.3]) in detectable fluorescence after 10-h incubation whereas the *Penicillium* yielded only a six-fold increase ([60.1 – 28.0]/[7.4 – 2.3])

(Table 3[i]). Given the apparent greater degree of development in evidence for *P. expansum*, this result suggests either that appreciably less free radicals, etc., are produced by the developing fungus or that this species has a more sophisticated apparatus for its protection against free radicals. In the light of the origins of this particular isolate, Antarctica, where it can be subjected to extremes of cold and increased levels of UV radiation, the latter alternative is possible. The contour plots provided in Fig. 2 show the relative values of fluorescent activity with and without the addition of the stains for *P. expansum* after 10-h incubation at 28 °C.

3.3. The impact of stress—mesophilic microfungi

To better gauge the applicability of flow cytometry to developing fungi, we introduced two other fungal species, *P. herbarum* and *T. viride.* To gain some measure of the impact that less than ideal growth conditions may have on mesophilic fungi, these species were incubated at reduced temperature (room temper-

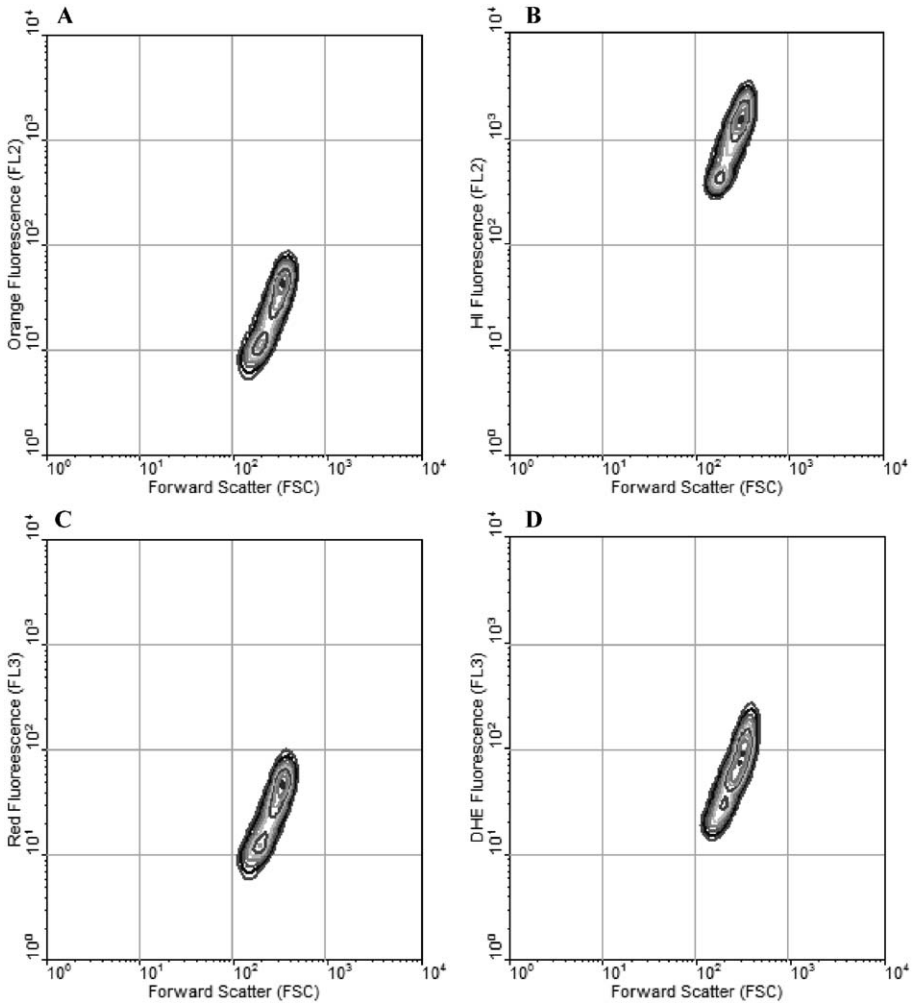

Fig. 2. Contour plots of *P. expansum* after 10 h incubation at 28 °C. The relative positions of unstained hyphae (A) and hyphae stained with HI (B) measured in the FL2 fluorescence channel and unstained (C) and DHE-stained hyphae (D) in the FL3 channel illustrate the amount of activity that can be identified using flow cytometry. All measurements are in arbitrary units (AU).

ature) with less vigorous agitation (lower oxygen) and stained with DHE to evaluate the impact of the increase in stress. For direct comparison, the Antarctic *P. expansum* was also cultured under these changed conditions.

Once again, the development of these fungi was observed using forward and side scatter and fluorescent activity (Table 3[ii]). Overall growth in the *T. viride* and the *Phoma* appeared reduced at room temperature when compared with *T. reesei* cultured at 28 °C with FSC values reduced from 200+ to around 100 AU (Table 3). Such a result was not surprising in view of the less than favourable growth conditions for mesophilic fungi at room temperature. The DHE fluorescence (in the FL3 channel) for *T. viride* at 17.1 AU represented 15% of its FSC value, which, although lower, did not differ markedly from that obtained for both *T. reesei* and *P. expansum* (20%). By contrast, the value obtained for the *Phoma* at 52.9 AU or 48% of FSC suggested that this species' stress response (i.e. its ability to produce antioxidants) was either overwhelmed in the face of a relatively higher level of stress or it is less well developed.

3.4. The Antarctic isolate

A very different picture emerged for the *P. expansum*. The cooler growth conditions appeared to have favoured this Antarctic isolate with values exceeding those previously obtained for its mesophilic analogue; FSC 340.4 cf. 256.9 AU, SSC 158.4 cf. 170.3 AU and DHE (FL3) 67.3 cf. 60.1 AU (Table 3[ii]). The measurable differences observed in the flow parameters suggested that flow cytometry has the potential to be exploited for the temperature screening of filamentous fungi. By manipulation of the growth conditions of parallel cultures, cold/heat adapted species could be identified.

The isolates were permitted to continue growing and were periodically analysed by the flow cytometer (not shown). It was observed that beyond 12–13 h of incubation, filamentation had developed to a degree that prohibited further examination. The time period during which analysis was practical varied between species and was dependent upon growth conditions. Therefore, actual timings would need to be determined empirically for each species.

4. Conclusions

We have identified a window of opportunity during which detailed analysis of filamentous fungi can be undertaken by flow cytometry. This ability to utilize flow cytometry as a tool to study the metabolic activity of filamentous fungi allows several possibilities. Applications currently employed by those investigating bacteria (see Veal et al., 2000) are potentially available for use with filamentous fungi. De Lucas et al. (1998) differentiated between ethanol-fixed propidium iodide (PI) stained haploid and diploid strains of *A. fumigatus* and Gourmet et al. (1997) also used PI to differentiate between fixed fungal species infecting plant tissue. Hopfer et al. (2001) were able to identify filament formation in heat treated *A. fumigatus* using ethidium bromide as the stain. However, in each of the examples cited, the method employed in the preparation of the fungal material was destructive. We have demonstrated that through the judicial choice of stains, the nondestructive screening and sorting of fungal material is possible.

In this investigation, we have shown that the introduction of hexidium iodide to the fungal cell permitted the flow cytometer to track the development of the fungi from germination through the growth of hyphae. We have also demonstrated, through the in vivo oxidation of dihydroethidium (DHE), that flow cytometry can be used to identify metabolic activity occurring within developing fungal hyphae. The formation of reactive oxygen species (ROS), a result of 'leakage' of electrons from the cellular electron transport chain, is an inevitable consequence of aerobic metabolism (Kreiner et al., 2002; Møller et al., 1996; Osiewacz, 2002). When DHE is introduced to an active cell, ROS will oxidize the stain to ethidium that can be identified by a flow cytometer when it intercalates with the DNA. Lledías et al. (1999) demonstrated that singlet oxygen was generated in conidia of *Neurospora crassa* at the onset of germination. Aerobic cells have evolved strategies for defense against damage induced by such species; however, under stressful conditions, oxidant levels may increase to overwhelm the antioxidants resulting in cell damage (Kreiner et al., 2002).

This capacity to isolate metabolic activity in germinating spores using cell permeant fluorogenic stains can be extrapolated to the identification of specific enzyme production within the growing cell. The use of

appropriate probes provides an avenue for screening populations of germinating spores for the presence of targeted enzyme activity. Not only can enzymatic activity be identified by selective staining but oligonucleotide and antibody probes, covalently linked to fluorescent organic compounds such as fluorescein isothionate (FITC), can be developed to target unique cellular characteristics.

An important feature of flow cytometry is its ability to analyse individually thousands of cells per second. Where a cell displaying a specific attribute is to be identified and selected (sorted), reduced flow rates are more practical. Even at a reduced rate of 300 events per second, these machines are capable of analysing and sorting around one million spores/germinating spores per hour. This ability to examine and select from such a vast number of events has the potential to significantly improve the chances of identifying particular fungal mutants and transformants within a much shortened time frame by obviating the need to undergo exhaustive screening on selection medium which is the current practice. The incorporation of genes carrying fluorogenic selection markers, such as green fluorescent protein (GFP) or its analogues, with the target gene construct could provide a convenient avenue for identifying genetically engineered transformants.

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RESEARCH ARTICLE

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The application of PCR for the isolation of a lipase gene from the genomic DNA of an Antarctic microfungus

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Abstract We successfully isolated a lipase gene (designated *lipPA*) directly from the genomic DNA of an Antarctic isolate of *Penicillium allii* using PCR and a suite of degenerate primers specifically designed to target two conserved regions of fungal lipase genes. We applied the biolistic transformation system to successfully integrate the *lipPA* gene into a heterologous fungal host, *Trichoderma reesei*, one of the most powerful secretors of extracellular proteins, and induced the transformant to secrete an active lipase into the growth medium. The recombinant lipase had a temperature optimum of 25 °C at pH 7.9 and retained greater than 50% of the maximum activity from 10 °C to 35 °C and over a pH range from 4.0 to 8.5.

Keywords Lipase · Antarctic fungi · PCR

Introduction

Lipolytic enzymes are ubiquitous in nature, being widely distributed throughout all kingdoms of life (Fojan et al. 2000). Fungi produce different classes of these enzymes, in particular carboxyl esterases (EC 3.1.1.1), which hydrolyse short-chain carboxylic acids (C ≤ 10; Sunna et al. 2002), and lipases (EC 3.1.1.3), which catalyse the hydrolysis and synthesis of long chain acylglycerides (C≥12; Eggert et al. 2000). The three-dimensional structures of a number of lipases and esterases have been elucidated and all have been shown to be members of the α/β hydrolase superfamily (Jaeger et al. 1999; Schmidt-Dannert 1999; Fojan et al. 2000; van Pouderoyen et al. 2001). The active site of α/β hydrolases contains a catalytic triad consisting of conserved serine, aspartic/glutamic acid and histidine residues (Jaeger et al. 1994, 1999; Brzozowski et al. 2000; Bornscheuer et al. 2002). Despite sharing a common catalytic mechanism and structure, lipases share little overall similarity with each other at the amino acid level (Brzozowski et al. 2000).

Lipases have applications in numerous industrial processes, such as the processing of oils and fats, detergent manufacturing, cheese making, baking, leather and paper processing. In addition, lipases are the most used enzymes in synthetic organic chemistry, catalysing the chemo-, regio- and/or stereoselective hydrolysis of carboxylic acid esters or the reverse reaction in organic solvents (Reetz 2002). The development of lipases based on technologies for the synthesis of novel compounds is rapidly expanding the use of these enzymes. The limiting factor, however, is a shortage of lipases having the specific required processing characteristics (Sharma et al. 2001). Many novel lipase genes are still to be identified and enzymes with new and exciting properties remain to be discovered. For these reasons, prospecting for novel lipase genes is of interest from both the industrial and academic standpoints.

Novel lipase genes can be difficult to isolate due to several factors, including toxicity of expression to heterologous hosts and a requirement for helper proteins to achieve functional lipase expression (Bell et al. 2002). In addition, the low homology observed between different lipase genes makes them difficult targets for a polymerase chain reaction (PCR). One strategy for obtaining new lipase genes is to sequence the N-terminal amino acid of a lipase and design a complementary degenerate oligonucleotide probe. A DNA library constructed from the lipase-producing organism can be screened using the lipase-specific

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oligonucleotide probe or the probe can be used to facilitate the construction of a mini-library enriched for the target lipase (Oh et al. 1999). Alternatively, a library constructed from a lipase-producing organism can be screened for recombinant bacteria expressing a functional lipase (Chung et al. 1991; Simons et al. 1998). The recombinants producing lipases can be identified by fluorescent indicators, such as rhodamine B (Kouker and Jaeger 1987). In the case of fungi, this latter strategy is further limited by the likely presence of introns in the target gene, making direct expression in a bacterial host most unlikely.

Bell et al. (2002) demonstrated that PCR could be used to amplify small regions of bacterial lipase genes directly from chromosomal DNA, using highly degenerate consensus primers and cloning a complete lipase gene from a mixed environmental DNA sample. Examination of sequence data in the region of the serine active site of fungal lipases (Bell et al. 2002) indicated a high level of conservation at the amino acid level. Another conserved site, corresponding to the oxyanion hole, was also identified in fungi (Herggård et al. 2000).

In our current work, we expanded the PCR approach to filamentous fungi. Lipase activity was clearly identified from an Antarctic isolate of *Penicillium allii* when it was grown on Rhodamine B indicator plates. We targeted two conserved sites as the most promising for the design of fungal PCR primers in order to isolate potential cold-active lipases from this Antarctic microfungus and to attempt their expression in a heterologous fungal host, *Trichoderma reesei*.

Materials and methods

Biological strains and plasmid

The native host of the lipase gene, *P. allii* DAR74613 (GenBank accession AF218787), was provided by Dr. R.D. Seppelt of the Australian Antarctic Division from a collection obtained from the Windmill Islands, continental Antarctica (66 ° 17′ S, 110 ° 32′ E). *T. reesei* VTT-D-79125, a high cellulase-producing mutant strain (Bailey and Nevalainen 1981), was used as the heterologous host for expression of the lipase gene. Fungal strains were maintained on potato/dextrose agar (PDA) plates. Plasmid pHEN54 (Bergquist et al. 2002), which harbours the strong cellobiohydrolase 1 (*cbh1*) promoter and bacterial hygromycin phosphotransferase (*hph*) gene as a selectable marker, was generated in this laboratory and utilised as the expression vector. The propagation of plasmids in *Escherichia coli* strain DH5α was performed using standard protocols, as described by Sambrook et al. (1989), and plasmids purified using Qiagen (Clifton Hill, Vic., Australia) columns according to the manufacturer's instructions.

Preparation of genomic DNA

Genomic DNA (gDNA) from *P. allii* was isolated directly from the mycelial mass grown on PDA, using the FastPrep FP120 bead-beating machine (Bio101, Calif., USA) in accordance with the manufacturer's directions. gDNA from *T. reesei* transformant colonies was similarly isolated after hygromycin selection and purification as described by Te'o et al. (2002).

Isolation of lipase gene fragments and genome-walking

P. allii gDNA (diluted 1:10, approx. 10 ng) was used as a template for the PCR amplification of lipase gene fragments, using degenerate consensus primers FoxF1-4 and FacR1-2 (Table 1) in all possible combinations and Ampli*Taq* Gold polymerase (Applied

Table 1 Oligonucleotides used in this study. Base abbreviations: *R* a or g, *Y* c or t, *M* a or c, *K* g or t, *S* g or c, *N* a, c, g or t

Function	Primer name	Primer attachment points (see Fig. 2)	Sequence (5′ → 3′)
Fungal lipase-prospecting primers (forward)Oxyanion hole	FoxF1		atc gtt ctg gYn KtN MgN gg
	FoxF2		att gtc ctt KcN KtN MgN gg
	FoxF3		att tac att KYN ttN MgN gg
	FoxF4	542–561	atc ggc atc RSN ttN MgN gg
Fungal lipase-prospecting primers (reverse)Active site	FacR1		tgc ccc tcc NaK Nga Rtg NSc
	FacR2	766–746	tgc gcc Ncc NaK Rct Rtg NSc
Genome-walking PCR primers	funlip1	644–667	cat cgt ggg ttc tgg gtt tat tgg
	funlip2	667–644	cca ata aac cca gaa ccc acg atg
	funlip3	1,009–1,032	ccg cca aac agc atc tac cga gcc
Primers for amplification of *Penicillium allii* lipase	explip3F	108–127	cag gca tct aga gcg gat ttg caa ttc agt cg
	explip3R	1,560–1,541	cag ata ctg cag cgc gtg atc act aca tga ag
Primers to delete introns	funlip6	245–265, 327–347	ctg tcc aac tag ccc gcc gag caa ttt caa acg agc tac tgg
	funlip7	347–327, 265–245	cca gta gct cgt ttg aaa ttg ctc ggc ggg cta gtt gga cag
	funlip8	800–820, 876–908	ggg gtt cac ttt gga tat ttg gac att tgg agg acc gaa acc
	funlip9	908–876, 820–800	ggt ttc ggt cct cca aat gtc caa ata tcc aaa gtg aac ccc
Primers to introduce restriction sites	explip4F	232–253	atc att gcg *tcg ac*t gtc caa c (*Sal*I in italics)
	explip6R	1,229–1,210	acg ta*g gta cc*t gca gtc agt ggt ggt ggt ggt ggt gac gag tag tag tag tag gtg atg atg cat ctg acg g (*Kpn*I in italics)
Primers to confirm integration	cbh1fseq		gtc aac cgc gga ctg ggc atc
	cbh1pfwd		gat tca gcg tac ccg tac aag tcg taa t
	cbh1term		gct acg ttg tca tcg tct tga cag caa tgc

Biosystems, Melbourne, Vic., Australia). PCR reactions (50 µl reaction volume) were performed using a GeneAmp PCR System 2400 thermal cycler (Perkin Elmer/Applied Biosystems) under the following conditions: one cycle of 95 °C for 15 min, followed by 40 cycles of 95 °C for 30 s, 53 °C for 30 s and 72 °C for 30 s, with one final cycle of 72 °C for 5 min. Fragments of approximately the expected size (~220 kb) were cloned into *E. coli* DH5α using the vector pCR2.1 (Invitrogen, Mount Waverly, Vic., Australia), suitable for blue/white assays. Plasmids from successful transformants were sequenced and two complementary oligonucleotide primers (funlip1, funlip2) were synthesised corresponding to a region selected from within the gene fragment. These, together with the primer funlip3, constituted the specific primers for genome-walking PCR (GWPCR) and are shown in Table 1. Linker assembly, linker library construction and GWPCR were performed according to Morris et al. (1995, 1998). The PCR reactions were performed as above, with the annealing temperature increased to 68 °C and the primer extension time extended to 3 min.

Amplification of full-length lipase gene

The full-length gene was isolated from *P. allii* gDNA by PCR, using the primers explip3F and explip3R (Table 1) under the following the conditions: one cycle of 95 °C for 15 min, followed by 40 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min, with one final cycle of 72 °C for 5 min. The PCR product was purified using the Concert rapid PCR purification system (Life-Technologies, Melbourne, Vic., Australia) in accordance with the manufacturer's instructions; and the sequence of the lipase gene was obtained by direct sequencing.

Excision of introns and preparation for transformation

Overlapping primer pairs, funlip6/funlip7 and funlip8/funlip9 were synthesised (Table 1) and, in combination with the primers explip3F and explip3R, introns identified in *lipPA* were excised by PCR. The same PCR conditions applied to obtain the full-length gene were used and the three resulting DNA fragments were gel-purified using QIAquick gel extraction kit (Qiagen). To generate an intron-free gene construct, the purified fragments (1 µl of each product) were subjected to a further round of PCR, using the two outer primers (explip3F/explip3R). The following PCR conditions were used: one cycle of 95 °C for 10 min, then ten cycles of 95 °C for 30 s, 70 °C for 30 s and 72 °C for 30 s, followed by 40 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 2 min, with one final cycle of 72 °C for 5 min. The resulting PCR product was gel-purified and confirmed by direct sequencing. Restriction sites *Sal*I and *Kpn*I were incorporated by PCR, using primers explip4F and explip6R (Table 1) to enable incorporation of the gene with the transformation vector pHEN54 (Te'o et al. 2002). PCR conditions, using *Taq* polymerase (Eppendorf), were: one cycle of 95 °C for 2 min, followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min, with one final cycle of 72 °C for 5 min. The PCR product was gel-purified and confirmed by sequencing.

Ligation of lipase gene (*lipPA*) and pHEN54

Both the plasmid pHEN54 and the *lipPA* gene PCR product containing no introns were double-digested with *Kpn*I and *Sal*I [Progen (MBI Fermentas), Qld., Australia] and purified using the QIAquick PCR purification kit. The two fragments were ligated using T4 DNA ligase (Roche Diagnostics Australia, Castle Hill, NSW, Australia) and the resulting construct was transformed into *E. coli*. Successful transformations were identified on LB-ampicillin selection plates and those containing the desired construct were identified by PCR using *Taq* polymerase and primers cbh1fseq and explip6R (Table 1) under the following conditions: one cycle of 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 2 min, with one final cycle of 72 °C for 5 min. The expression construct (pHEN54lip; Fig. 1) was sequenced to confirm that the fusion was in-frame and no PCR errors had been introduced.

Biolistic transformation of *T. reesei* conidia and PCR tests for integration

Biolistic bombardment of *T. reesei* conidia with pHEN54lip followed the method detailed by Te'o et al. (2002), using rupture disks with a strength of 9,300 kPa and a target distance of 3 cm. Integration of *lipPA* within the genome was confirmed by PCR, using a forward primer, cbh1pfwd, positioned within the cellobiohydrolase 1 promoter region (*cbh1p*) in combination with the reverse primer funlip2 (Table 1; Fig. 1). To establish whether pHEN54lip had successfully integrated at the *cbh1* locus, PCR reactions were performed using the same forward primer (cbh1pfwd) in combination with the reverse primer cbh1term (Table 1), which was designed to anneal at the extreme 3′ end of the known *cbh1* terminator region and was not present

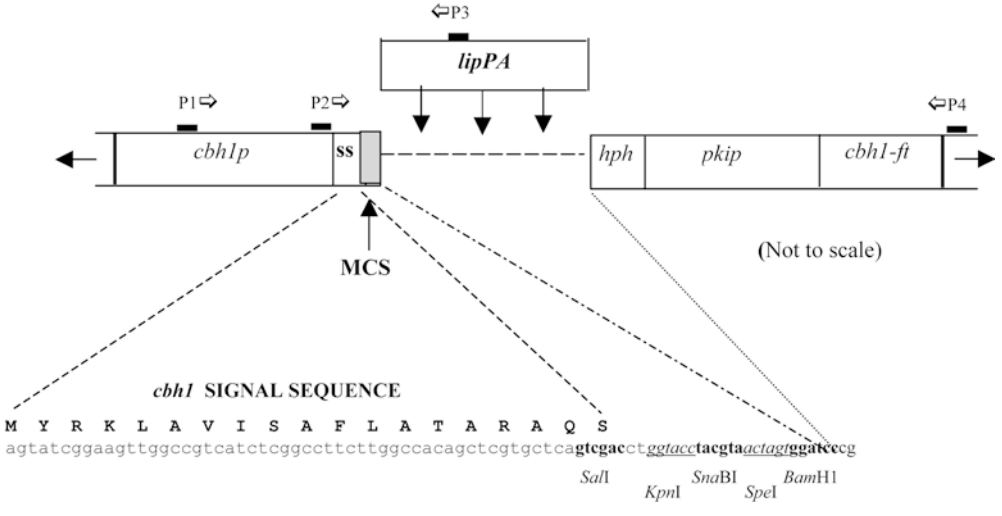

Fig. 1 Schematic of the 5.5 kb expression construct of pHEN54lip. The *lipPA* gene is fused to the *cbh1* signal sequence retained from pHEN54. The attachment site of primers used to confirm integration and determine whether recombination has occurred at the *cbh1* gene locus are indicated as: *P1* cbh1pfwd, *P2* cbh1fseq, *P3* funlip2 and *P4* cbh1term. *cbh1p* Cellobiohydrolase promoter from *Trichoderma reesei*, *hph* bacterial hygromycin phosphotransferase, *pkip* pyruvate kinase promoter from *T. reesei*, *cbh1*–ft full terminator (less ~50 bp) of *T. reesei cbh1*

on the *cbh1-ft* fragment contained on the transformation construct (Fig. 1). PCR reactions were carried out using *Taq* polymerase with these conditions: one cycle of 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 2–3 min, with one final cycle of 72 °C for 5 min.

DNA sequencing

DNA was sequenced by the dideoxy chain-termination method (Sanger et al. 1977) using the ABI PRISM BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems) and was analysed on an ABI 377 automated sequencer.

Culture conditions for lipase production

Untransformed *T. reesei* and selected transformants were cultured on PDA plates until well conidiated (~10 days). Fresh conidia were harvested by washing with sterile saline solution (0.9%) containing Tween 80 (0.01%) and were filtered through sterile cotton wool to remove any residual mycelia. Conidia ($\sim 10^7$) from each isolate were inoculated into 50 ml of growth medium containing KH_2PO_4 (0.75 g), $(NH_4)_2SO_4$ (0.25 g), Avicel (1 g), soy hydrolysate (0.75 g), 20% lactose (2.5 ml), 1 M $MgSO_4$ (121 µl) and 1 M $CaCl_2$ (204 µl). Cultivation was carried out in Erlenmeyer flasks (250 ml) at 28 °C on an orbital shaker at 250 rpm. The supernatant was harvested after 6 days and freed from the fungal mycelia by centrifugation at 10,000 *g* for 10 min. The centrifuged supernatant was further clarified using a PD-10 column (Amersham Biosciences, Castle Hill, NSW, Australia) and was buffer-exchanged by elution in PBS (pH 7.0). The supernatant was stored at 4 °C until assayed.

Lipase activity assay and characterisation of the enzyme

Lipase activity was determined using the *p*-nitrophenyl esters of fatty acids as substrates (Sigma–Aldrich, Castle Hill, NSW, Australia). The standard assay reaction mixture contained 40 mM *p*-nitrophenyl caprate (C_{10}), 120 mM universal buffer (pH 8.0; Britton and Robinson 1931) and undiluted culture supernatant (enzyme); and the total reaction volume was 400 µl, in the ratio 20:340:40. The reaction mixture was incubated at room temperature (23–24 °C) for 1 h and the reaction was stopped by the addition of 200 µl of 1 M Na_2CO_3. The change in absorbance was measured at 405 nm against a non-reacted blank. The supernatant from untransformed *T. reesei* was similarly assayed as a control. Enzymatic activity under the experimental conditions was calculated using a molar absorption coefficient of 14,800 M^{-1} cm^{-1}. Substrate specificity towards different *p*-nitrophenyl esters (C_4–C_{18}) was determined as described for the standard assay conditions.

The enzyme (supernatant) was assayed at pH values ranging from 4 to 10 (120 mM universal buffer) at room temperature for 1 h. The effect of temperature on the reaction rate was determined by incubating the enzyme at temperatures ranging from 10 °C to 50 °C at pH 8.

Protein determination

Protein concentrations were determined with the BCA protein assay kit (Pierce, Rockford, Ill., USA), using bovine serum albumin as a standard.

Database-searching and analysis

The BLASTP service available at the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/) was used to determine alignments with the lipase gene. Prediction of likely signal sequence cleavage sites was performed using the SignalP ver. 2.0 web server (http://www.cbs.dtu.dk/services/SignalP-2.0).

Results and discussion

Design of lipase-prospecting primers

We applied the strategy described by Bell et al. (2002) to design degenerate PCR primers targeting the two conserved regions identified in fungal lipases. Several factors make the design of PCR primers using these sites difficult. These include the moderate level of homology, the presence of unfavourable amino acids (serine, arginine, leucine) within the conserved sequences and the shortness of the conserved sites. Using PROSITE (http://au.expasy.org/prosite) nomenclature (in which ambiguities are indicated by listing the acceptable amino acids for a given position between square brackets ″[ ]″ and each element in a pattern is separated from its neighbour by a dash ″-″), the consensus sequence of the active-site region can be defined as G-H-S-L-G-[GA] and that of the oxyanion hole as [IV]-[VY]-[LVI]-[AVS]-F-R-G. A detailed examination of the data for filamentous fungi indicated that the distance between these two sites was consistently around 72 amino acids (216 bp).

The most commonly used strategy to isolate distantly related sequences by PCR is to design degenerate primers which bind to highly conserved regions of DNA sequence. We have applied the CODEHOP primer design principles (Rose et al. 1998) to design a set of two-part primers, consisting of a consensus clamp and a degenerate core, complementary to the active site and oxyanion hole (Table 1). The degeneracy of the primers went slightly beyond the maximum level generally accepted for PCR (Rose et al. 1998).

Isolation of lipase gene

When the prospecting primers were applied to *P. allii* genomic DNA, a number of bands were obtained which included several of the expected size (220 bp). After cloning and sequencing a 225-bp DNA band generated by the primer combination FoxF4 and FacR2 (Table 1), it was found (upon translation to amino acids) to display some homology with known lipase genes. GWPCR (Morris et al. 1995, 1998) was used to obtain the full-length gene sequence (Fig. 2). The sequence associated with the lipase gene, designated *lipPA*, revealed the presence of two introns (61 bp, 64 bp), shown in lower case in Fig. 2. Each intron exhibited the consensus sequence (underlined in Fig. 2) for the 5′ splice site (GTPuNGPy), the 3′ splice site (PyAG) and the branch site (CTPuAPy; Toida et al. 2000). Excision of the introns yielded an open reading frame encoding 308 amino acid residues. The *lipPA* sequence was submitted to BLASTP and the results obtained (Table 2) indicated that this sequence showed maximum alignment with the filamentous fungal lipases available in the database.

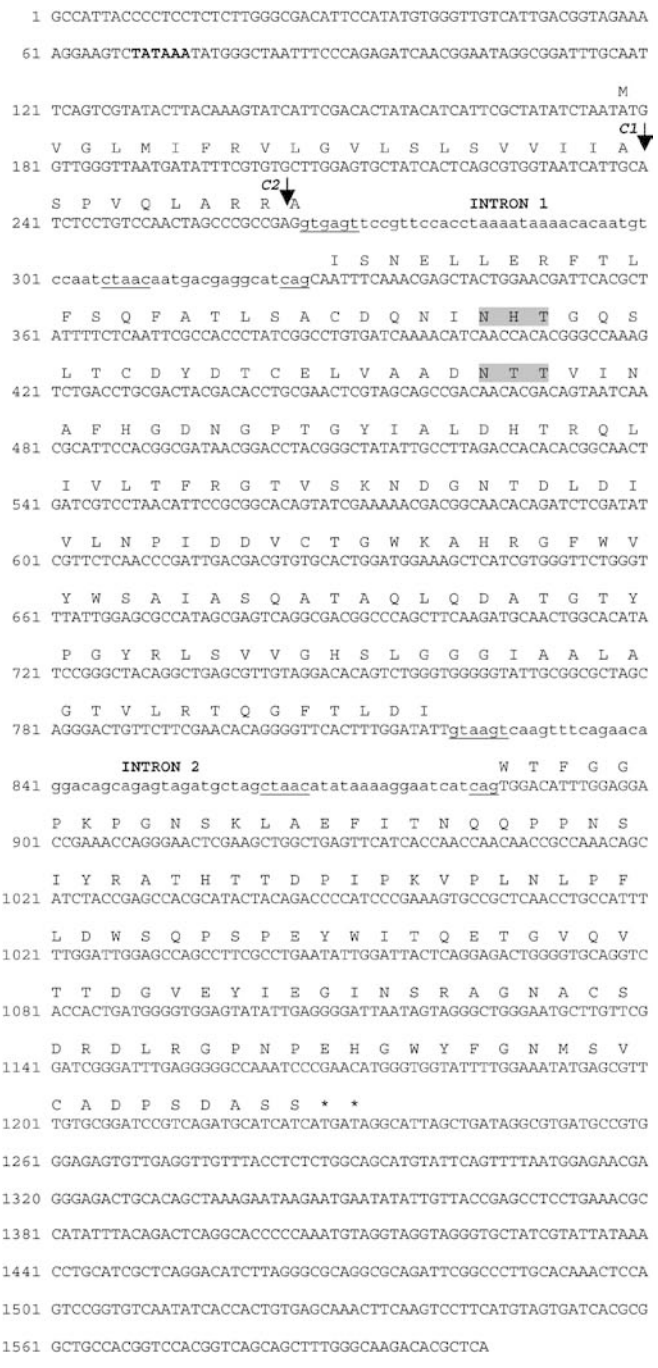

Fig. 2 Full LipPA sequence showing amino acid sequence deduced from the putative lipase gene *lipPA*. The two possible cleavage sites of the signal peptide sequence, *C1* and *C2*, are *indicated by arrow heads* and possible N-glycosylation sites are *highlighted by shadowing*. Introns are *shown in lower case* with the consensus sequence for the 5′ and 3′ splice sites and branch site *shown underlined*. The sequence data reported here was submitted to GenBank and assigned accession number AY303124

Features of the protein encoded by *lipPA*

As our primary search was for extracellular lipases, the presence of a signal peptide at the N-terminus of the protein could indicate the likelihood of a secreted enzyme. The *lipPA* sequence data was submitted to the sequence analysis program SignalP (see Materials and methods) in order to obtain a statistical estimate for the presence of a signal peptide and to identify the probable cleavage site. The neural networks model (Nielsen et al. 1997) located a most likely cleavage site between residues 21 and 22: IIA-SP (indicated by arrow *C1* in Fig. 2). An estimate using the more recently developed hidden Markov models (Nielsen and Krogh 1998) predicts a cleavage site between residues 29 and 30: ARR-AI (arrow *C2* in Fig. 2) which coincidentally synchronises the N-terminus of the mature protein with that of its closest homologue, triacylglycerol lipase of *Thermomyces lanuginosus*.

Table 2 Results of BLASTP search for sequence homology, showing the percentage (with absolute numbers in parentheses) of residues in the lipase sequence of the listed fungi which are identical (*Identities*) with *lipPA* and those amino acids (overall) for which the physico-chemical properties of the corresponding *lipPA* residue are preserved (*Positives*)

Organism	Accession number	Identities	Positives
Thermomyces lanuginosus	O59952	37% (104/280)	57% (160/280)
Penicillium camembertii	BAA14345	34% (96/275)	49% (136/275)
Aspergillus oryzae	BAA12912	34% (97/284)	48% (139/284)
A. flavus	AAO17921	34% (97/284)	48% (139/284)
A. parasiticus	AAO17920	34% (96/281)	49% (138/281)
Neurospora crassa	CAC28687	31% (85/274)	51% (140/274)
Fusarium heterosporum	AAB34680	29% (82/274)	47% (129/274)
Nectria haematococca	CAC19602	30% (82/273)	47% (130/273)

DNA analysis of *Trichoderma reesei* transformants

Biolistic transformation of *T. reesei* conidia yielded 29 hygromycin-resistant transformant colonies. PCR analysis performed on the transformants revealed that integration of the complete 5.5-kb cassette (Fig. 1) had occurred in 17 of the 29 transformations (data not shown). To establish whether integration had occurred at the targeted *cbh1* locus, PCR was performed with the gDNA of the 17 successful transformants (data not shown), using the extreme primers (see Materials and methods). A single transformant, T16, yielded a product of the correct size, indicating integration at the desired locus. The bulk of the protein secreted into the culture supernatant by the host *T. reesei* strain is attributable to the main cellobiohydrolase I protein. The sharp decrease in total secreted protein by transformant T16 (about 50% of that of the untransformed host) is further indirect evidence of integration of the cassette in the *cbh1* locus (data not shown).

Synthesis of recombinant lipase in *T. reesei*

It has been demonstrated that heterologous genes can be expressed by *T. reesei*, using the strong *cbh1* promoter (Paloheimo et al. 1998; de Faria et al. 2002). In this work,

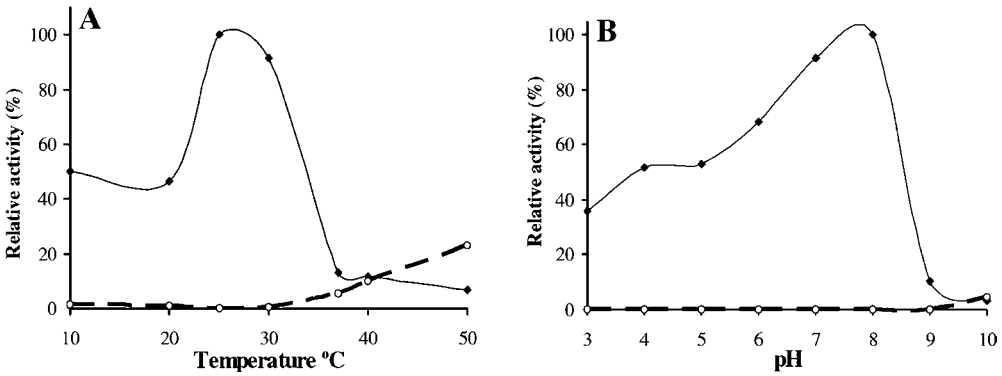

Fig. 3A, B Effect of temperature and pH on the enzyme activity of recombinant LipPA produced in *T. reesei*. **A** The effect of temperature was measured whilst holding pH constant at 8.0. **B** pH activity was measured at room temperature. The activity of LipPA is *shown by solid lines* and that of untransformed *T. reesei* is *shown by broken lines*

each of the 17 successful transformants (together with the untransformed *T. reesei*) was grown in liquid culture to induce lipase production under the control of the *cbh1* promoter. As transformant T16 appeared to have the expression cassette integrated into the *cbh1* gene locus (data not shown), we concentrated initially on this strain. The recombinant lipase (LipPA) was assayed in the culture supernatant of T16 using *p*-nitrophenyl caprate (C_{10}) as substrate. The enzyme was active between 10 °C and 50 °C, with an optimum temperature for activity at 25 °C. Although 50% of its activity was retained at 10 °C, this reduced quickly above 30 °C (Fig. 3A). Assays at room temperature (23–24 °C) indicated that optimum activity occurred at pH 7.9 (Fig. 3B), retaining 50% at pH 4.0. Activity was observed to fall dramatically above pH 8.0 (Fig. 3B). At the optimum temperature and pH, enzyme activity was 58 nkat mg^{-1} total protein. Supernatant from the untransformed host was assayed in parallel with LipPA and failed to show any activity towards the substrate under any conditions. Assays were also conducted with *p*-nitrophenyl laurate (C_{12}) as substrate (data not shown) and these yielded similar results to those above, albeit at a reduced level of activity (see below).

Lipases have been defined as hydrolysing long-chain acylglycerols (≥10 carbon atoms; Sunna et al. 2002). However, it is recognised that they are also capable of hydrolysing glyceroesters with a shorter acyl chain length. Assays undertaken for the recombinant using a range of nitrophenyl esters indicated a preference for C_4–C_{10} esters over C_{12}–C_{18} esters (data not shown). The reduced activity towards the longer-chain fatty acid substrates may reflect the enzyme's difficulty in accessing the substrate rather than an inherent lack of affinity. Under the experimental conditions (room temperature), the long-chain esters tend to precipitate in the buffer solution, significantly limiting the amount of substrate that can interface with the enzyme. Notwithstanding this possible constraint, measurable levels of activity were obtained for the long-chain esters.

Conclusion

In this study, we demonstrate that PCR methodology can be used to prospect for novel fungal lipase genes directly from chromosomal DNA, despite the low homology observed between lipases. Since the PCR methods described allow the specific amplification of rare sequences within the complex DNA mixture, many of the problems associated with isolating lipase genes using library-based methods can be overcome. The *lipPA* gene isolated in this investigation shows sufficient predicted amino acid and structural homology with known fungal lipases for us to conclude that it represents a true lipase of *P. allii*.

A comparison of the activity profile identified for the recombinant LipPA lipase with those published for commercially available fungal lipases indicates the potential novel nature of this Antarctic protein. An extensive list of data supplied by manufacturers to Godfrey and West (1996) shows that the pH optima of commercially available lipases range from as low as pH 5 for a number of products through to pH 11 for a genetically modified *Thermomyces lanuginosus* lipase expressed in *Aspergillus oryzae* (Lipolase; Novo Nordisk). The temperature optima for the commercial products vary from 30 °C (at pH 7) for Amano Pharmaceutical's Lipase R (from *P. roquefortii*) through to a number of products in the range 45–55 °C. In contrast, the Antarctic *P. allii* lipase expressed in *Trichoderma reesei* indicates a temperature optimum of 25 °C at pH 7.9 and retains 50% of its activity at 10 °C and also at pH 4.

We conclude that the PCR method described here is useful for prospecting for potentially novel lipase genes in filamentous fungi. In combination with the biolistic transformation of a suitable fungal host, novel enzymes can be expressed by their heterologous host into the growth medium and assayed directly.

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