

# **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 60<sup>th</sup> 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,  $\beta$ -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 10°C and as much as 30°C lower than that of the reference strain and that mannanase activity for two of the Antarctic isolates exceeded 40% of their maximum at 0°C. 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.

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# 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- $\beta$ -D-thiogalactosidase
ITS	internal transcribed spacer region
Kbp	kilo base pairs
$k_{cat}$	dissociation rate ( $s^{-1}$ ) - <i>Michaelis-Menten kinetics</i>
kDa	kilo Dalton
$K_M$	Michaelis constant ( $mol\ L^{-1}$ ) - <i>Michaelis-Menten kinetics</i>
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- $\beta$ -D-galactosidase