CHAPTER 1

Introduction

The origins of taxonomy

Aristotle (4 B.C.) classified all living organisms as either animals or plants. Although this was the first documented evidence of such classification there is little doubt that the idea predates Aristotle. Linnaeus (1707-1778) classified organisms this way as well, but also included a kingdom he called Chaos, in which he placed all microorganisms. Linnaeus a Swiss Botanist, was the first to propose a formalised naming system that he referred to as binomial nomenclature. The binomial nomenclature comprised a generic (genus) and specific (species) name. Linnaeus also proposed that all naming should be in Latin as this language was no longer in use and it would prevent ambiguity based on the native language of the researcher. With such an organised system of hierarchical order in place, researchers were able to study populations in a logical sequence understanding relationships among taxons. The two Kingdom classification of Aristotle was challenged in 1866 by Ernst Haeckel (1834-1919). Influenced by Charles Darwin's theory of evolution, Haeckel introduced the Kingdom Protista comprising of all microscopic, single-celled organisms, but his proposal was largely ignored by the scientific community. In 1956, Herbert Copeland (Margulis, 1998) proposed a four Kingdom system comprising of Animal, Plant, Protoctists and Bacteria. The elevation of the eukaryotes and prokaryotes from phyla (in Haeckel's Protista) to Kingdom demonstrated the recognised evolutionary differences between bacteria and single-celled eukaryotes. Shortly after in 1959, Robert Whittaker, an American ecologist, proposed a fifth Kingdom, the Fungi, once again in recognition of the differences between the already accepted groups (Whittaker, 1969). However, Whittaker's work did not receive much recognition until a revised version was published in 1970 by Lynn Margulis (1998):

- Animalia
- Plantae
- Fungi
- Protista (Protoctista)
- Monera (the prokaryotes)

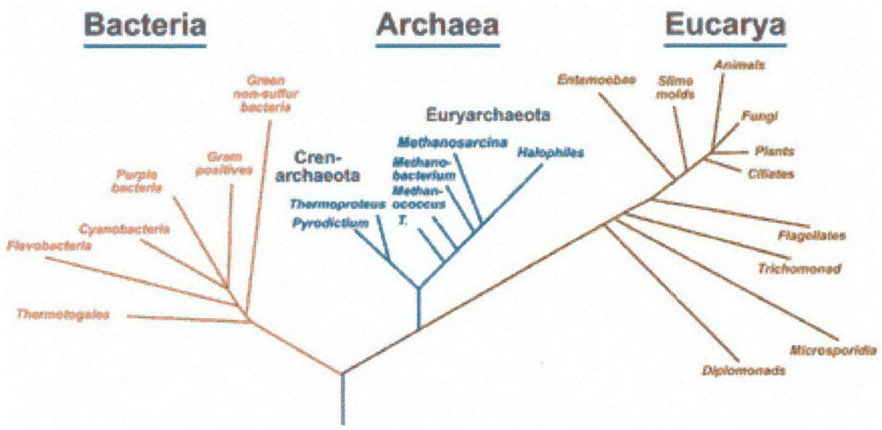


Figure 1.1. Phylogenetic tree showing evolutionary distances among the three domains, *Archaea, Bacteria* and *Eucarya*. (Woese, 1994).

This five Kingdom system has been the most widely recognised and accepted version. In 1990, the most extensive revision of Whittaker's system was made by Carl Woese. Based on molecular studies of the ribosomal RNA (rRNA) of prokaryotes and eukaryotes, Woese found that the Archaebacteria differed significantly in the rRNA gene sequences from both the other groups but there were no significant differences among the eukaryota themselves. Woese proposed a higher order, Domain, of which there were three categories; Eukarya, Bacteria and Archaea. Within the Domain Eukarya are the four eukaryote Kingdoms of Whittaker [Fig 1.1]. Carl Woese was awarded the Leeuwenhoek Medal by the Dutch Royal Academy of Sciences in 1992 for his research. According to Woese (1990):

> *"The central task of biology in the new century will be to lay out and elaborate this overarching framework of relationships among living organisms.... This endeavor will help us to understand how the essential unit of all life, the cell, came into being. It will help us to understand the evolutionary interactions among microbial species that gave rise to, sustain, and have the potential to drastically alter the nature of our biosphere."*

Table 1.1: Comparison of classification schemes

2 Kingdom System	5 Kingdom System	3 Domain System While Kingdom is still used, the concept is of diminished relevance	
	Kingdoms	**Domains**	**Kingdoms**
Plantae	Monera (the prokaryotes)	Archaea	Estimates range from 18 to 30 or more
		Eubacteria	Unclear
	Plantae	Eukarya	Fungi
	Fungi		Plantae (algae?)
	Protoctist (e.g. algae, diatoms and different plankton)		Protista
Animalia	Animalia		Animalia

(Colour key- prokaryotic, eukaryotic, mixed)

Kingdom Fungi

The Domain Eukarya (also called Eumycota or true Fungi) can be classified into four phyla: Chytridiomycota, Zygomycota, Ascomycota and Basidiomycota [Fig 1.2]. Most fungi are multicellular with the exception of the unicellular yeasts. Fungi are heterotrophs with chitin as the major cell wall component, as compared with plant cell walls consisting of cellulose. Fungi are nonphotosynthetic and are eukaryotic.

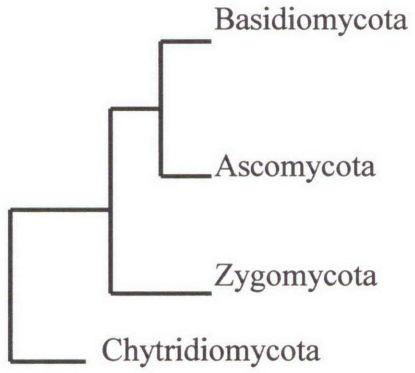


Figure 1.2. Unrooted phylogenetic tree showing the relative positions of fungal clades

Threadlike filaments of walled multinucleate hyphae extend apically and combine to make up the mycelium. Reproduction can be either sexual or asexual. They are widely distributed throughout nature but occur mainly terrestrially and are ecologically important as decomposers with some fungi forming mutually beneficial associations with plants.

The dual capacity of reproduction, i.e. sexual and asexual, has resulted in a dual nomenclature. The sexual state is known as a teleomorph and the non-sexual state(s) as the anamorph (Barnett *et al.*, 1990). Developing at different stages they are often described without knowledge of the other, resulting in separate binomials. The vegetative anamorphs have been grouped together in a separate taxon (deuteromyces) as they share some morphological features. Often referred to as Fungi Imperfecti (with the teleomorph referred to as Fungi Perfecti), these fungi have no known means of sexual reproduction, and include genera such as *Penicillium*, *Aspergillus, Saccharomyces* and *Candida*. The deuteromycetes are an artificial assemblage of fungi that biologists have placed in a single phylum for convenience, as modes of sexual reproduction have historically been the basis for fungi classification. Molecular techniques have revealed deuteromycotina to be polyphyletic throughout many of the fungal clades. With the advent of molecular techniques, anamorphic species are being placed amongst their phylogenetic relatives resulting in the recognition of evolutionary relationships.

The inclusion of the phylum Chytridiomycota has attracted criticism because these organisms possess flagella and have, therefore, been included in the Protoctista. However, cell wall components and the ability to synthesize lysine have placed them in the phyla Fungi (Guarro *et al.*, 1999). Sequence analysis of 18S rRNA indicates closer phylogenetic relationships between the Chytridiomycota and both the Ascomycota and Basidiomycota than the Protists. Chytridiomycota form the basal branch in the fungal phylogenetic tree and it has been well documented that the Zygomycota diverged first, with the Ascomycota and Basidiomycota forming sister clades [Fig 1.2] (Bruns *et al.*, 1992).

Definition and classification of Fungi

A diverse range of fungi within the Ascomycota and Basidiomycota have a distinct vegetative stage known as the yeast cell. Yeasts cells are unicellular and may result from budding of a meiospore (ascospore or basidiospore). In some cases, the entire life cycle takes

budding of a meiospore (ascospore or basidiospore). In some cases, the entire life cycle takes place in the unicellular stage. In contrast, some fungi are dimorphic, that is they have two distinct life stages, one unicellular and the other filamentous. Dimorphic Basidiomycetes are distributed throughout the three classes of Basidiomycota: Urediniomycetes, Ustilaginomyctes and Hymenomycetes. Sexual species reproduce by budding of the meiospore followed by conjugation between two sexually compatible yeast cells giving rise to a dikaryotic mycelium, which in turn will form the sexual structures. Dimorphic Basidiomycetes exhibit some common traits such as: the haploid stage corresponding with the unicellular stage; the unicellular stage resulting from basidiospore germination; the parasitic stage (when parasitic) corresponding with the filamentous (sexual) stage; and the unicellular stage corresponding with the saprobic stage (Boekhout *et al.*, 1998).

Ascomycota

The vast majority of described fungal species fall into either the phyla of Ascomycota or Basidiomycota. Ascomycota comprise almost 80% of all known fungal species and has received the most attention as the majority of pathogenic fungi belong to this phylum. Some of the most notable species being *Candida albicans* and *Cryptococcus neoformans*, both of which are human pathogens, species of *Aspergillus* and *Penicillium* and the baker's yeast *Saccharomyces cerevisiae*. The Ascomycetes have historically been placed into two classes, the Hemiascomycetes and the Euascomycetes. Hemiascomycetes are characterized by asci that do not form within a fruiting body (ascocarp), while Euascomycetes form asci either within or upon a fruiting body (Kurtzman, 1998a). Current classification systems have included a third class, the "Archiascomycetes", as a result of phylogenetic analysis of molecular sequences (Kurtzman, 1998a).

Many Ascomycetes are responsible for fungal diseases in agriculture, food spoilage and humans. For example, *Drechslera maydis* was responsible for destroying corn crops in the USA in 1970 and *Penicillium italicum* causes the mould often found on oranges. *Aspergillus flavus* grows on peanuts producing a mycotoxin (aflatoxin), which contaminates food and causes liver damage even at very low concentrations and is regarded as a potent carcinogen. Superficial fungal diseases in humans (mycosis) range from ringworm of the scalp to athlete's foot and are caused by keratin attacking species of *Microsporon* and *Trichophyton*.

On a more positive note, Ascomycetes are crucial in their role as nutrient recyclers that break down organic material in soil. There are also many genera that live in symbiosis with algae forming lichens and some plants depend upon those species that form mycorrhizae with their roots. In the food industry, enzymes from *Penicillium camembertii* and *P. roquefortii* are responsible for the texture and flavour of cheeses such as Camembert, Brie, Roquefort, Danish Blue and Stilton. *Aspergillus oryzae* turns soya protein into soy sauce and ketchup not to mention the use of *S. cerevisiae* in beer and bread production. *Candida famata,* is an over-producer of riboflavin (vitamin B_2) and *Yarrowia lipolytica, C. zelanoides* and *C. citrica* are capable of producing high yields of citric acid. Several species of *Pichia* and *Aureobasidium* produce extracellular polysaccharides and ascorbic acid is produced by *C. norvegensis* (Demain *et al.*, 1998).

In the health industry, *P. chrysogenum* was one of the most potent weapons against bacterial disease, producing the penicillins. More recently, cyclosporine (isolated from the mold *Tolypocladium niveum*), has been identified as the most effective and least toxic immunosuppressant yet discovered, dramatically improving the success rate of organ transplants as well as being used as a treatment for several autoimmune diseases such as multiple sclerosis, aplastic anaemia and Addison's disease (Isaac *et al.*, 1990; Brody *et al.*, 1994; Rosenfeld *et al.*, 2003).

Basidiomycota

The Basidiomycetes are divided into three major classes: the Urediniomycetes consisting of the smuts, the Ustilaginomycetes composing the rusts and the Hymenomycetes containing both the Heterobasidiomycetes, where the jelly fungi can be found, and the Homobasidiomycetes which comprises the mushrooms (Swann and Taylor, 1995). The most recognized of the Basidiomycetes are the mushrooms. *Agaricus* is the common edible mushroom. The genus *Amanita* includes some of the most deadly and mystic mushrooms such as *Amanita muscaria*. The genus *Lycoperdon* identifies the puffballs, very large edible fungi with enclosed basidiocarps known as glebas. Other fungi belonging to the basidiomycetes include the coral fungi, *Clavaria*, so named because of their resemblance to some forms of coral. In agriculture, the smuts, *Ustilago*, are parasites of several important crops such as wheat, oats and rye that cause significant economic impact. The rusts are divided into two

groups based on their life cycles. Autoecious rusts complete their life cycles on a single host, while heteroecious rusts require at least two different species of hosts. All are serious pests of plants, but the heteroecious forms include some of the most devastating to agricultural crops. *Puccinia graminis*, wheat rust (also known as red stem rust and black stem rust) is the most notorious of the genus, responsible for extensive damage to wheat crops. Its complex life cycle includes five types of spores and involves two hosts - wheat, *Triticum* and barberry, *Berberis vulgaris*.

There are many industrial applications of Basidiomycota species including *Phaffia rhodozyma* as a source of astaxanthin, the main carotenoid present in this species. This orange-red pigment is used as a colour additive in aquaculture, in egg yolks and in the commercial production of crustaceans (α-carotene formation by *Rhodotorula glutinis* and L-phenylalanine production by *R. rubra*). Live cultures of *Cr. laurentii* have been used to control grey mold, a disease affecting apple trees (Demain *et al.*, 1998). Many species are being exploited for the enzymes they produce, including xylanases from *Cr. albidus*, cellulases from *Cr. cellulolyticus* and amylase activity in *Cr. tsukubaensis, Cr. curvatus* and *Filobasidium capsuligenum* (Fell *et al.*, 2001).

Yeasts

Yeasts are unicellular fungi. Historically, the taxonomy of unicellular fungi evolved separately from other areas of mycology, mainly due to the role that yeast play in the fermentation industry. Yeasts have traditionally been assigned to genera based on morphological characteristics, such as vegetative and sexual states. To date, numerous taxonomic techniques have been utilized for yeast identification including classical techniques such as carbon assimilation and fermentation tests, growth temperature tests and cellular morphology (Barnett *et al.*, 1990). These tests are not only laborious, but also offer diminutive reliability due to strain variability (Kurtzman and Robnett, 1998). Moreover, results from these classical methods often do not indicate phylogenetic relatedness resulting in the reclassification of many species (Fell *et al.*, 2000).

The term 'yeast' in the past has had various meanings. 'Yeast' was (and occasionally still is) used to designate a taxonomic group. This definition is outdated and clearly incorrect as yeasts do not form a natural group, but are polyphyletic throughout the Ascomycota and

Basidiomycota. The term 'yeast' has also been used as a definition for the strictly unicellular ascomycetous yeast, with 'yeast-like' reserved for those unicellular fungi with a filamentous stage or those that produced dark pigments (such as *Aureobasidium* spp.). These definitions are problematic and vary between authors.

Yeasts can be found in a variety of habitats from fresh to marine waters, in soils from tropical islands to the polar regions, and in association with plants, animals and humans. However, yeasts are generally limited to where they can be found due to the significant amounts of organic carbon required for metabolism. Hence, they are often associated with moist habitats, which allow for prompt nutrient absorption. They are capable of growth over a wide range of pH values but are limited in their ability to grow at high temperatures with few yeasts capable of growth above 45°C (Lachance and Starmer, 1998).

Microorganisms, including yeast, may be classified according to their temperature growth range. Temperature is one of the most important factors governing the growth and survival of all organisms. The term thermophile (Greek [Gk.] *thermos*, hot), mesophile (Gk. *mesos*, middle) and psychrophile (Gk. *psukhros*, cold) have been used extensively for the classification of microorganisms into distinct thermal domains. However, in the case of yeasts, these terms require modification. The term thermophile is usually used for organisms that have an optimum growth temperature of >45°C and the term hyperthermophile reserved for those with an optimum growth temperature >80°C (Brock, 2000). Strictly speaking, there is no such organism as a thermophilic yeast, given that the maximum growth temperature of yeasts is around 48°C (Stokes, 1971). The term thermophile, therefore, as it applies to yeasts requires modification and it has been proposed that this term be applied to yeasts that are unable to grow below 20°C, ie. minimum growth temperature of 20°C with no limitation applied to the maximum growth temperature (Watson, 1987). The term psychrophobic (Mendonca-Hagler and Phaff, 1995) has also been proposed for yeasts that fall into this category. The term psychrophile is used to describe organisms whose optimum growth temperature is <20°C. Some differentiation has come about between an obligate psychrophile and a facultative psychrophile, with the latter having a maximum growth temperature that is above 20°C (Ingraham and Stokes, 1959). There is no restriction on the minimum growth temperature due to the difficulty in accurately determining such a parameter as a result of the inhibitory effects of the addition of antifreeze to the growth media at temperatures below 0°C. Other terms that

have been used interchangeably are psychrotolerant and thermophobic, indicating growth at >25°C but with an optimum growth below 20°C (Watson, 1987). The term mesophile has been traditionally used for those organisms whose growth range is between 10°C and 48°C. An alternative definition for yeasts has been proposed by Watson (1987) as *'those who do not fall into one of the other two domains'* (i.e. thermophile or psychrophile). By this broad definition, the vast majority of yeasts are classified as mesophiles, with a growth temperature range between 0°C to 48°C.

These characteristics define the niche that yeasts inhabit. Yeasts do not occur randomly throughout nature, but rather form communities, and are thereby defined by their habitat (Lachance and Starmer, 1998). The physiological properties of a yeast species will determine its environmental specificity. However, those properties are not necessarily accurate at predicting where a particular yeast species may be found.

Yeasts are best known for the role that they play in food production, namely bread, beer and wine. Although *S. cerevisiae* is responsible for most beer production, other yeast species such as *Candida, Brettanomyces* and *Saccharomycopsis* are utilized in many countries that use traditional techniques (Fleet, 1993). As with beer, *S. cerevisiae* is the primary yeast used in wine production, although most wine fermentations are the result of a complex ecology of such species as *Candida, Pichia, Kluyveromyces, Metschnikowia*, *Schizosaccharomyces* and *Zygosaccharomyces* (Fleet, 1993). Yeasts are also used in the production of such cheeses as Limburger, Tilsitter, Port Salut, Trappist and Danish Danbo. These cheeses are matured by a method known as surface ripening. Yeasts are part of a complex microflora present on the cheese surface and induce the ripening process by raising the pH thereby allowing the growth of crucial bacteria (Petersen, 2000). Yeasts are incorporated in the production of smallgoods such as salamis with *Yarrowii lipolytica* and *Debaryomyces hansenii* used as starter cultures contributing to improved flavour and the reddening reaction (Fleet, 1993; Suzzi *et al.*, 2000).

Other industrial uses for yeasts include the commercial production of citric acid, riboflavin, astaxanthine and phenylalanine, the control of fruit spoilage by antagonistic activity towards filamentous fungi, the production of breads and sour dough (Fleet, 1993), and in single cell protein fodder production (Raspor *et al.*, 2000).

Fungal Taxonomy

The study of taxonomy is to understand the relationships of living organisms in evolutionary terms. If two (or more) species are members of the same genus, then it is recognized that they have a common ancestor. These relationships are represented as clades. When a speciation event occurs, then two new clades are produced. If a species in the new clade evolves into two species then two new clades are produced within the original clade. When a group of related clades are mapped together they form a phylogeny. This phylogenetic tree thus provides the evolutionary history of a particular group.

The classification system of fungi has historically been based on morphological characteristics such as cell wall structure and sexual reproduction. This approach has been widely criticized due to its lack of stability and subjectivity. This is particularly pertinent when some phenotypic characteristics are dependant upon environmental conditions. Laboratory conditions can only mimic natural habitats and interactions between other organisms are not studied during species identification. Consequently, many species of fungi will not be identified correctly using morphological criteria.

The basic status of taxonomy is the species. Based on Linnaean rank designations, taxa are ranked from Kingdom down to genera. This method has been superceded by the concept of rank-free classification due to the volume of clades produced. Linnaean ranks are still used, but are reserved as an indication of biological classification. A summary of Linnaean rank designations is presented in Table 1.2.

Table 1.2: Classification of Fungi

Domain	Eukarya
Kingdom	Fungi
Phylum	-mycota
Class	-mycetes
Order	-ales
Family	-aceae
Genus	-
Species	-

Fungi were originally classified as Plantae under the system of Aristotle following the definition of Animalia being mobile and Plantae immobile. Even following the five Kingdom classifications, fungi were still considered to be more closely related to the Plant Kingdom rather than the Animal Kingdom. This has since been revised as studies of cellular structures and physiology revealed that many of the so-called "lower Fungi", such as the slime molds and oomycetes, are not related to the fungi at all. The one exception was the chytrids which were thought to be the basal line of the fungi. It is now believed that the Animal and Fungi Kingdoms share a common ancestor, a flagellate with similar characteristics to the chytrids.

Nomenclature

Historically, nomenclature has been inconsistent. The Kingdom Protista is used interchangeably with the older version Protoctista. The terms Basidiomycetes and Ascomycetes are used in the context of phylum instead of the correct "mycota" suffix. The term "phylum", used by zoologists has all but replaced the botanical term "division," again illustrating the shift away from botany. In the CD-ROM programme, "Yeasts of the World", the terms division and sub-division are used rather than Domain and Phylum respectively (Boekhout *et al.*, 2002). The use of an assortment of nomenclature by mycologists and zymologists highlights the inconsistencies of fungal taxonomy, thus making it a difficult area of study to circumnavigate for the new generation of researchers.

The *International Code of Botanical Nomenclature* governs the nomenclature of fungi. As stated previously, a species must be given a Latin binomial name consisting of a generic name (the genus) and a specific epithet (species name). For a new species to be recognised, it must be published in an internationally recognised journal along with a Latin description of essential characteristics, including features that distinguish it from previously accepted species. Failure to comply with these standards results in an invalidly described species (*nomen invalidum*). An original specimen must be deposited in an internationally recognised and accessible herbarium and is designated the "Type strain". The naming of taxa must follow the rules of Latin derivation, including gender designations (Kurtzman and Fell, 1998).

Identification & classification methods

Accurate identification of fungal species is paramount not only in the clinical setting, but also in plant pathology, biotechnology and environmental studies. Traditionally, yeasts have been classified and identified using observable physiological features such as cellular morphology, biochemical tests, fatty acid composition, cell wall composition, protein composition and ubiquinone systems. These phenotypic analyses to identify yeasts have proved to be ambiguous and questionable, thus leading to long lists of synonyms for some taxa (Kurtzman and Phaff, 1987; Fell *et al.*, 2000). The introduction of molecular methods has not only accelerated the classification process, but has also simplified it. Identification of new taxa is relatively rapid and accurate using state-of-the-art computer software designed to compare DNA sequences of large numbers of species. Such techniques have enabled the development of systematics thus enabling rapid identification and more comprehensive phylogenetic analyses.

Morphology

The nature of the cell wall can differentiate between ascomycetous and basidiomycetous species. Electron microscopy is used to examine the budding site. In basidiomycetous yeasts, budding results in a collar-shaped scar due to the rupturing of the multi-layered cell wall, whereas ascomycetous yeasts have two appressed cell layers (Moore, 1998). The biochemical test using diazonium blue B (DBB) supports this dichotomy with ascomycetes not responding to DBB but basidiomycetes responding with a red to purple colour. Asexual reproduction in yeasts occurs by budding, fission or by the production of conidia. Budding can occur at one pole of the cell, termed monopolar, at both poles (bipolar) or from various sites (multipolar). Fission is the duplication of a cell by a septum bisecting along the long axis. Conidia formation is uncommon among the yeast, however, the way in which the conidia are formed (conidiogenesis) can differentiate between species such as *Sterigmatomyces* and *Fellomyces* (Moore, 1998).

Identifying the type of fruiting body present is also used for classification. Varying types of asci can be classified according to shape (globose, saccate, cylindrical, hat-shaped, saturnoid, spindle-shaped), the number of ascospores present (two to four are the most common but there can be many more), and the size and colour. Teliospores in basidiomycetous

yeasts are also of various shapes (globose, ovoidal or angular) and can be pigmented, as is the case in *Rhodotorula toruloides* (Yarrow, 1998; Guarro *et al.*, 1999).

Physiological and biochemical techniques

Physiological and biochemical techniques are still used to identify yeasts. Characteristics such as maximum growth temperature, the ability to utilize certain carbon or nitrogen compounds and the ability to ferment carbohydrates as well as colony formation are the most common properties used in yeast identification.

The ability of a yeast to grow aerobically on a particular organic compound as the sole source of energy is routinely used in identification. These compounds include sugars, alditols and organic acids. On average, fifty tests are commonly used in yeast taxonomy, this equates to a highly laborious and time-consuming method for identification purposes when often time is a limiting factor. More recently, automated techniques have been developed to detect whether a yeast can assimilate a certain compound by using a dye (tetrazolium violet) resulting in a positive reaction recorded as purple. Standard microplates with 96 wells are used allowing for 95 compounds plus a negative control to be tested simultaneously. Computer software programs coupled with a microplate reader provide automated results (Robert *et al.*, 1997). In the clinical setting, there are commercially available kits for the identification of commonly known human pathogens such as *C. albicans* (Bujdakova *et al.*, 2004; Deak *et al.*, 2004; Marot-Leblond *et al.*, 2004; Melkusova *et al.*, 2005).

Fatty acid composition

It has long been established that the fatty acid composition of cell membranes are important for an organism's ability to function at low temperatures (Watson, 1987). This has produced research into the effects of fatty acid composition on membrane fluidity, in particular the ratio of saturated versus unsaturated fatty acids. Watson & Arthur (1976) investigated the fatty acid composition of three psychrophilic yeasts (*Leucosporidium frigidum, L. gelium, L. nivalis*) isolated from Antarctica and found a predominance for C_{18} monounsaturated and polyunsaturated fatty acids. Although cellular fatty acid composition has been used extensively in bacterial taxonomy (Veys *et al.*, 1989), it has only received recognition in fungal taxonomy quite recently (Augustyn *et al.*, 1990; Brondz and Olsen, 1990; Amano *et al.*, 1992). Gas

chromatography combined with statistical analysis has been used to study fatty acid composition among various fungi including basidiomycetes, oomycetes and zygomycetes (Guarro *et al.*, 1999). Standardisation of this method is required as variations in growth temperatures and growth media can alter fatty acid profiles (Watson, 1980).

Cell wall composition

There are significant variations in structure and composition of fungal cell walls and capsules. Ascomycetous yeasts have a low chitin (1-2%) content that is mainly confined to the bud scar region. In comparison, basidiomycetous yeasts have a much higher chitin content that is evenly distributed throughout the lateral walls (up to 10%). However, these studies have been confined to a limited number of yeast genera, *S. cerevisiae* in the case of the ascomycetes and species of *Cryptococcus, Rhodotorula* and *Sporobolomyces* of the basidiomycetes (Phaff, 1998). It may, therefore, be anticipated that there would be many exceptions to the rule.

Protein composition

The total cellular protein composition of an organism is a reflection of its DNA. Although not all proteins are expressed at any given time, under constant conditions (e.g. temperature and media) the expression of proteins should remain consistent. Several studies have used one-dimensional-sodium dodecyl sulphate polyacrylamide gel electrophoresis (1D-SDS-PAGE) of whole cell extracts to construct protein profiles of various organisms (Vancanneyt *et al.*, 1992; Vandamme *et al.*, 1998). In the case of yeast species commonly associated with humans, such as *C. albicans, C. glabrata, C. guilliermondii, C. krusei, C. parapsilosis, C. pseudotropicalis, C. tropicalis, Geotrichum candidum* and *S. cerevisiae*, 1D-SDS-PAGE of whole cell extracts have been able to clearly distinguish among fifty-two strains from these nine species with no differences detected between strains from the same species (Bruneau and Guinet, 1989).

Vancanneyt *et al.* (1992) compared the protein profiles of representative strains from the basidiomycetous yeasts. Using the Pearson product moment correlation coefficient or similarity value (r), these authors were able to delineate species qualitatively and quantitatively by comparison of the protein profiles produced. Variation between strains of the same species ranged from 0.84 to 0.96, thereby indicating a similarity value (r) for conspecific

strains of ≥ 0.85. All of the species examined demonstrated characteristic whole cell protein profiles with the exception of the four *Mrakia* species. *M. frigida, M. gelida, M. nivalis* and *M. stokesii* which displayed uniform protein profiles with *r* values ≥ 0.85 suggesting that all four *Mrakia* species are synonymous (Vancanneyt *et al.*, 1992). It has since been established that *M. stokesii* is a synonym of *M. gelida* and *M. nivalis* is a synonym of *M. frigida* on the basis of variations observed in the ITS and IGS regions of the ribosomal rRNA (Diaz and Fell, 2000).

Electrophoretic comparison of enzymes have been used extensively in systematic studies of ascomycetous and basidiomycetous yeasts (Yamazaki *et al.*, 1998). A large variety of enzymes have been used including fructose-1,6-bisphosphate aldolase, lactate dehydrogenase, fumarase, catalase and superoxide dismutase to name just a few. Although accepted as an economical and practical method when screening large numbers of species, no clear relationships regarding taxonomical hierarchy can be inferred (Yamazaki *et al.*, 1998).

Coenzyme Q systems

Coenzyme Q (ubiquinone, CoQ) is an essential component of the mitochondrial respiratory chain in eukaryotic cells. Coenzyme Q has been used as a taxonomic tool in yeasts due to the wide structural variation within different taxonomic groups and the relative ease with which it can be isolated and characterized.

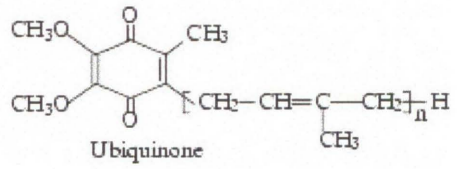


Figure 1.3. Diagrammatic representation of coenzyme Q (ubiquinone) where *n* = number of side chain isoprenoid units

The coenzyme Q homologs are expressed as Q-*n*, with *n* denoting the specified number of isoprenoid units in a side chain [Fig 1.3]. The structural differences in the coenzyme Q homologs have been used in the classification of genera as well as at the generic level for various organisms (Yamada *et al.*, 1989; Yamada, 1998; Guarro *et al.*, 1999; Suzuki and Nakase, 1999). Coenzyme homologs are tissue dependant, for example, the major coenzyme Q found in the rat is CoQ-9, however, in rat brain tissue CoQ-10 is the major homolog found. In bacteria homologs CoQ-6, CoQ-7, CoQ-8 and CoQ-10 can be found while for mammals, birds

and fish, CoQ-10 is predominant (Battino *et al.*, 1990). In the case of yeasts, the coenzyme Q homologs range from CoQ-5 through to CoQ-10. Historically it is used as a classification tool at the generic level as the length of the isoprene chain is usually consistent within a monophyletic group (Kuraishi *et al.*, 2000). The identification of the principal coenzyme Q homolog present periodically correlates with results provided by molecular techniques, although conclusions based on ubiquinone systems alone are contentious, closely related species can be separated by this technique.

Molecular techniques

Identification and classification of yeast species from classical assimilation tests and morphology have been problematic as these properties may be strain variable and often do not reflect genetic relatedness. This has resulted in relatively high frequencies of misidentifications. Although useful in the past for taxonomic studies, the carbohydrate assimilation tests are labour intensive with results often ambiguous in nature and slow in return. Molecular techniques are much faster, more accurate and have therefore been increasingly developed and utilized for yeast identification.

Molecular analysis has had a major impact on yeast systematics. The development of the polymerase chain reaction (PCR) allowed for the analysis of limited cell numbers such as dried herbarium samples or extinct organisms thus providing comprehensive phylogenetic studies. However, the first molecular method to be used in yeast systematics was the determination of the nuclear DNA (nDNA) guanine-plus-cytosine content (G + C). Variations of 2.0 - 2.5% in the G + C values inferred different species (Kurtzman, 1998b). The G + C content of ascomycetous yeasts is generally 27 - 50%, whereas the G + C content for basidiomycetous yeast is usually 50 - 70%. Inevitably, there was overlap between unrelated species thereby making the taxonomic use of G + C content exclusionary.

There are various methods for evaluating DNA relatedness. Percent relatedness is the most common way to express DNA complementarity. As a general rule, strains showing 70% or greater nDNA relatedness are considered conspecific while those with less than 40% nDNA relatedness are considered independent species (Kurtzman, 1998b; Guarro *et al.*, 1999). DNA reassociation kinetics has revealed the synonymy of a large number of species thus indicating

that many previous morphological and physiological characteristics used to define a species were phylogenetically insignificant (Kurtzman, 1998b).

There are a number of other methods used to identify phylogenetic relationships among species of fungi, such as restriction fragment length polymorphisms (RFLP), used to evaluate anamorph-teleomorph associations (Guarro *et al.*, 1999) and amplified fragment length polymorphisms (AFLP) to study genetic variation (de Barros Lopes *et al.*, 1999). These techniques are a simple and inexpensive alternative to rDNA sequencing when general relationships have already been established.

Ribosomal complex

There are many genes used in phylogenetic studies of fungi, but genes encoding the ribosomal complex are by far the most frequently used. Ribosomal DNA (rDNA) sequences have been used to assess close and distant relationships among fungi for a number of reasons. Firstly, ribosomes are essential cellular components that have been highly conserved throughout evolution, thereby providing a reference point from which various species and strains can be compared. Secondly, ribosomes appear to originate from a common ancestral line thereby providing a measure of evolutionary relationships (Kurtzman and Robnett, 1998).

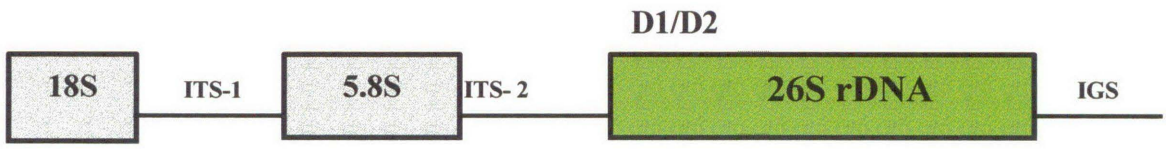


Figure 1.4. Arrangement of ribosomal genes in yeast

The ribosomal complex is a multi-copy, non-protein coding gene. Repeat copies are found in tandem with repeats comprising of three coding genes; 18S, 5.8S and 26S. Each cluster is transcribed along with internal (ITS) and external transcribed spacers (ETS). These spacers are spliced out during transcription. Separating each cluster is a non-transcribed (NTS) or intragenic spacer (IGS) [Fig 1.4] (Guarro *et al.*, 1999).

5S rDNA

The 5S rDNA, comprising approximately 160 nucleotides, is the smallest of the ribosomal genes. Due to the small number of nucleotides and its highly conserved nature, sequencing of this gene is easily determined, therefore, it was widely used for phylogenetic studies. Comparison studies of 5S rRNAs from basidiomycetous yeast confirmed the significance of a number of morphological features such as hyphal septa and spore morphology as well as establishing broad phylogenetic relationships (Kurtzman and Blanz, 1998). Relationships among ascomycetous yeasts indicated that the ascomycetes consisted of the three lineages, *Schizosaccharomyces* and *Protomyces*, the budding yeasts and filamentous fungi. Despite promising phylogenetic results, the 5S rDNA gene is limited by its highly conserved nature and thus inability to distinguish between closely related species and has thus been superseded by data obtained from the larger and more informative ribosomal genes (Kurtzman and Blanz, 1998).

18S rDNA

The 18S rDNA, also called the small subunit rDNA (SSU rDNA), has been widely used to analyse close and distant phylogenetic relationships of fungi as it contains regions of sequence divergence. Analysis of the 18S rDNA has been instrumental in establishing relationships among the eukaryotic kingdoms as well as defining the barriers of the fungi kingdom itself (Hasegawa *et al.*, 1985). Swann & Taylor (1995) identified three lineages within the basidiomycetous yeasts based on comparison of 18S sequences: the Urediniomycetes, the Ustilaginomycetes and the Hymenomycetes. Phylogenetic analysis of 18S rDNA indicated that ascomycetous yeast were polypheletic with spore discharge being lost convergently from three lineages of ascomycetes (Berbee and Taylor, 1992). The small subunit rDNA has been extensively used in the analysis of the filamentous fungi (Berbee and Taylor, 1992; Bruns *et al.*, 1992; Berbee, 1996) as well as the black yeast-like fungi (Haase *et al.*, 1995; Vitale and de Hoog, 2002) but a comprehensive database inclusive of all classes of fungi is lacking.

26S rDNA

The large ribosomal subunit (LSU 26S rDNA) consists of both conserved and divergent regions. Phylogenetic analysis using the 26S rDNA has supported genetic

relationships inferred from analysis of the 18S rDNA, however, they have also shown greater resolution of terminal lineages. The 5' end of the 26S rDNA molecule is quite variable and, with few exceptions, the D1/D2 region is sufficiently variable to identify closely related species. In the case of yeasts, the D1/D2 variable regions are widely used for taxonomic and phylogenetic analysis (Kurtzman and Robnett, 1998). Kurtzman and Robnett (1998) analysed this region from type species of all cultivatable ascomycetous yeasts as well as yeast-like species. The results indicated that ascomycetous yeasts form a sister clade to the filamentous ascomycetes (Euascomycetes) and supported the observations that many morphological features, such as budding, hold little phylogenetic significance. According to Kurtzman and Robnett (1998) strains showing greater than 1% divergence in this domain are likely to be different species while strains with 0-3 nucleotide differences are either conspecific or sister species. To validate these predictions, nDNA relatedness was examined. Findings indicated strains with less than 30% nDNA relatedness in general had greater than three nucleotide differences thus supporting their recognition as separate species.

In a similar study where over 300 strains of basidiomycetous yeasts were examined in the same D1/D2 region, there were no nucleotide substitutions in multiple strains within a species (Fell *et al.*, 2000). Results from this study indicated that strains differing by two or more nucleotides represented separate species, suggesting that the D1/D2 region may be more conserved within the basidiomycetous yeasts than in the ascomycetous yeasts. In some cases, where sequence data indicated conspecific species whilst phenotypic analyses suggested distinctly separate species, clarification was achieved by analysing the more variable ITS region.

Phylogenetic analysis

Phylogenetics is the study of the evolutionary relationships among organisms. Based on the taxonomic concepts of Haeckel and Whittaker, organisms are grouped together based on characteristics that they have in common. The basis of phylogenetics (as is the case in taxonomy) is the species. However, what defines a species has historically stimulated much debate. In 1963, the evolutionary biologist Ernst Mayr proposed the Biological Species Concept (BSC). This concept is based upon reproductive isolation:

"Groups of actually or potentially interbreeding natural populations which are reproductively isolated from other such groups." (Mayr, 1963)

This system works well for organisms that reproduce sexually, but is not a satisfactory system for those that reproduce asexually, such as binary fission in bacteria, fragmentation in algae and cyanobacteria and budding in yeast. There are also many exceptions among those organisms that reproduce sexually. One notable example is species of dingoes, though geographically isolated, can produce fertile offspring with other species. These violations led to the proposal of other concepts, one of which is the Phylogenetic Species Concept (PSC) proposed by Hennig. The PSC designates organisms as species according to diagnosable clusters of characteristics:

"... the smallest groups that are consistently and persistently distinct and distinguishable by ordinary means."

Apart from the obvious problem defining *ordinary means*, the PSC has similar difficulties to the BSC defining asexual organisms as well as hybrids. There are several variations to the PSC, one of which states a species must be monophyletic and share one or more derived characters. This concept too is problematic as many species of yeast can be identified by unique characteristics.

The Evolutionary Species Concept is defined as:

"A single lineage of ancestral descendant populations of organisms which maintains its identity from other such lineages and which has its own evolutionary tendencies and historical fate" (Wiley 1978)

However, this concept fails to identify which traits are most relevant as well as difficulties in deciding what constitutes a common evolutionary fate.

The ultimate goal of phylogenetics is to produce phylogenetic trees. These trees can then be used to test theories concerning the evolution of groups of organisms from a common ancestor, as well as aiding in the rapid and accurate identification of species. The evolutionary distance can be calculated using a wide diversity of available algorithms. Software programs designed to align sequences are employed to infer relationships based on the matrix produced using homologous regions that act as reference points. Trees are usually rooted with a moderately distant species, termed the out-group. Once the data is aligned, trees can be calculated by a variety of methods. The neighbour-joining method is a widely used and relatively fast algorithm that clusters sequences by minimising the sum of branch lengths with the results viewed as either cladograms or phylograms (Chenna *et al.*, 2003). Constructed trees are statistically analysed using resampling algorithms such as the bootstrap method. Trees are usually resampled from 100 to 1000 times with a majority-rule consensus tree produced.

The largest and most comprehensive analyses of the Basidiomycota employed the MEGALIGN (DNAstar) program to align sequences and results phylogenetically analysed with PAUP 4.0 (Swofford, 2001). It was therefore decided that, for consistency, these programs would be used in this study.

Antarctica

Antarctica is the fifth largest of the seven continents with a location almost entirely south of latitude 66°30'. Land mass is 14 million km^2 comprising of approximately 280,000 km^2 ice-free and 13.72 million km^2 ice-covered regions. Remote and hostile, it is the ultimate test of survival. Average elevations are between 2000 and 4000 meters with the mountain regions reaching almost 5000 meters. Compounding the temperature extremes, the interior of Antarctica is classified as a desert with very low snowfalls and an annual precipitation of 50 mm. Katabatic winds (caused by air that cools over the ice surface becoming heavier than surrounding air) blow towards the coastline from the elevated interior with blizzards occurring regularly near the plateau. Cyclones form over the ocean moving clockwise along the coast. Precipitation is much heavier along the coastal regions amounting to more than 200 mm yr^{-1}. Less than 2% of Antarctica is free from ice and these areas are restricted to coastal regions including parts of southern Victoria Land, Wilkes Land, parts of Ross Island on McMurdo Sound and the Antarctic Peninsula. The icecaps hold close to 70% of the worlds freshwater and 90% of the world's ice. In the winter months, the continent doubles in size due to freezing ocean waters. Glaciers form along coastal areas and floating icebergs comprise 11% of the area (http://www.cia.gov/cia/publications/factbook/geos/ay.html, 2004).

Gondwana, a landmass comprising of the southern continents, fragmented into separate pieces over 150 million years ago. The remnants of this event can be found in the rocks of the Antarctic, which contain traces of flora and fauna fossils. Within the layers of ice is an archive of historical climatic and microbial importance. One of the best examples is that of Lake Vostok, discovered in central Eastern Antarctica underneath the ice (ranging from 400 m to 4150 m), and which has evolved for millions of years, isolated from external environmental conditions (Kapitsa *et al.*, 1996). Estimates suggest that a climate record of over 400,000 years is locked away within the overlaying ice sheet. Variations in gas composition, dust concentration and stable isotope content have uncovered four complete climatic cycles related to four glacial periods (ice ages) and subsequent warming periods (Petit *et al.*, 1999). Core samples (whose ages are over 240,000 years old) taken to a depth of 1500 to 2750 m have revealed a plethora of microorganisms, including both bacteria and fungi (both filamentous and unicellular). Thawed samples from these ice cores assimilated ^{14}C-amino acids, establishing bacterial cells which have remained viable for 110,000 to 240,000 years (Abyzov

et al., 1998). Ancient Arctic bacterial isolates have been shown to grow at temperatures as low as -4.5°C, thus the low temperatures of -3.2°C of Lake Vostok should not be a limitating factor for microbial existence. However, renewable energy supplies will be due to isolation from exogenous sources of carbon and solar energy (Tiedje, 1998). The collaboration of Russian, American and French researchers have allowed drilling to a depth of 3623 m, the deepest ice core ever retrieved but drilling has since ceased until aseptic conditions can be assured so as not to contaminate this pristine environment. The latest media reports have indicated a renewal of drilling in the coming austral summer of 2005.

The McMurdo Dry Valleys, located on the western coast of McMurdo Sound (77°00'S 162°52'E) is home to the US research base. The soils are saline, coarse and periglacially active, with organic carbon content and moisture very low (Campbell and Claridge, 1987). Such extreme conditions impose severe limitations on all life forms and are considered to be at the end of the environmental limits by the National Science Foundation's Long-term Ecological Research (LTER) Program (http://lternet.edu/sites/mcm/, 2005). Dominated by microorganisms, mosses, lichens, and relatively few groups of invertebrates (tardigrades, rotifers and nematodes), higher life forms of plants and animals are virtually non-existent elevating the invertebrates to the top of the food chain. Lacking these variables, the soils of the Antarctic are unique even though microbial utilization and re-mineralization of nutrients are the same processes as is the case for any ecosystem. Minor alterations in climate can have a dramatic effect on biological processes such as microbial reproduction and growth with data generated by the National Science Foundation (NSF) indicating this region to be very sensitive to solar irradiation and temperature alterations. While it has long been recognised that climate change affects the ice-sheets of Antarctica, these affects take time. In comparison, climate changes can be seen almost immediately in the glaciers and lakes of the McMurdo Valley (http://lternet.edu/sites/mcm/, 2005). Recent research by the British Antarctic Survey (BAS), the US National Science Foundation and the University of Texas have revealed rapid thinning of two major glaciers in the Amundsen Sea sector thus speculating as to rises in sea levels (British Antarctic Survey Press Releases, 2005). At a recent conference, the director of the BAS made the following statement:

> *"Satellite measurements tell us that a significant part of the West Antarctic ice sheet in this area is thinning fast enough to make a significant contribution to sea level rise, but for the present, our understanding of the reason for this*

change is little better than hypothesis. The last IPCC report characterised Antarctica as a slumbering giant in terms of climate change. I would say that this is now an awakened giant. There is real cause for concern." (British Antarctic Survey Press Releases, 2005)

With research (spanning decades) into the depletion of the ozone layer over Antarctica, this recent report continues to highlight the impact of the global warming hypothesis and the thinning of the ozone layer, therefore, understanding of this delicate ecosystem is of prime importance.

Investigators began exploration of the Antarctic in the early 1800's and since the International Geophysical year of 1957, many nations have established scientific stations. In 1961, the Antarctic Treaty suspended all territorial claims by Argentina, Australia, Chile, France, NZ, Norway, and the UK and stated that the entire continent be dedicated to peaceful scientific investigation. The US and Russia have made no claims and do not recognise those made by other nations. In 2003, there were 45 treaty members; 27 consultative nations comprising of the seven claimant nations plus 20 non-claimant nations and 18 non-consultative nations. Administrative decisions are made by the consultative nations and carried out by those nations in accordance with their laws and operatives. There are a number of articles within the treaty, a few of the key ones are as follows:

- Freedom of scientific investigation
- Free exchange of information
- Does not recognize, dispute, or establish territorial claims and no new claims shall be asserted while the treaty is in force
- Jurisdiction over observers and scientists by their own states

Since the original treaty, governments have ratified some 200 recommendations implemented at consultative meetings. The protocol on Environmental Protection came into effect in 1998 with five specific strategies:

- Environmental impact assessment
- Conservation of Antarctic fauna and flora
- Waste disposal and waste management
- Prevention of marine pollution
- Area protection and management

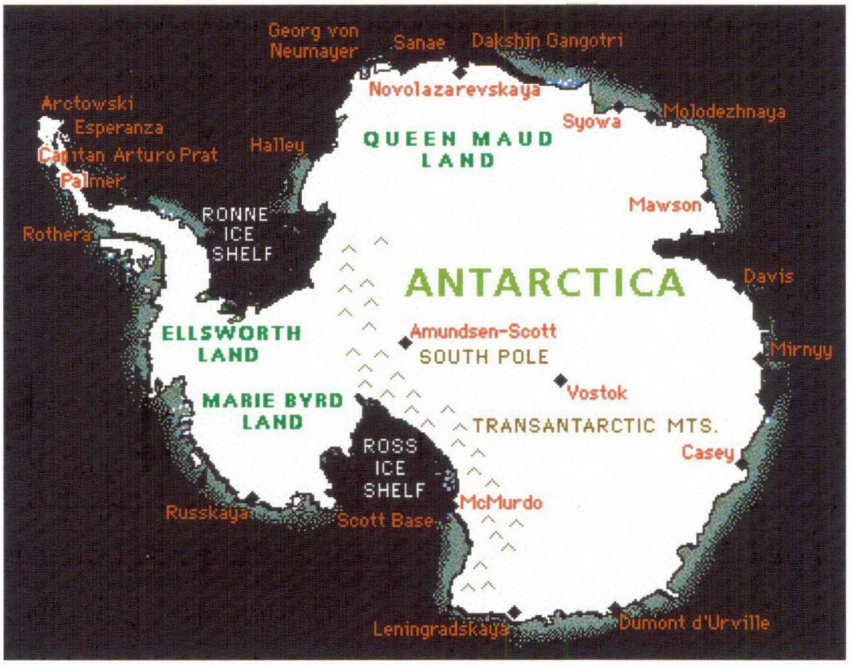


Figure 1.5. Map of Antarctica
http://www.theinternetradio.com/antarctica/antarc.htm

The Vestfold Hills

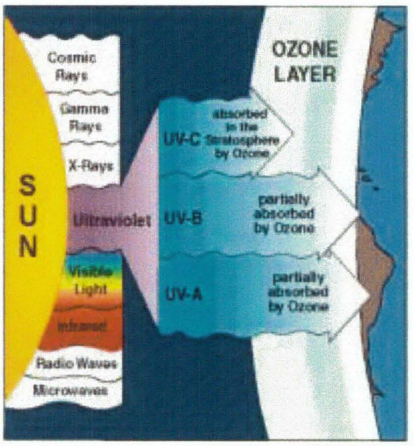
The Vestfold Hills is comprised of ice-free mainland rock and off-shore islands covering an area of approximately 512 km^2. Located on the Ingrid Christensen Coast of Princess Elizabeth Land (latitude 68° 33' 00.0" S, longitude 78° 15' 00.0" E), the hills average between 30 and 100 m above sea level with the highest peak reaching 159 m. Captain Klarius Mikkelsen named the Vestfold Hills after a county in Norway while on a Norwegian whaling expedition in 1935. Phillip Law for the Australian National Antarctic Research Expeditions (ANARE) carried out exploration in the austral summer of 1954/1955 and the Australian Davis Base station was established soon thereafter in 1957. Davis is one of three Australian stations (Mawson and Casey the other two) and supports the largest population of research scientists during the summer season [Fig 1.5]. Situated on the edge of the Vestfold Hills it is bounded by the steep ice-covered hills some 24 km east from the coast, the Særsdal Glacier to the south and by the sea to the northwest. The Hills region has an area of about 400 km^2 and is dotted with lakes of varying salinity (Australian Antarctic Division, 2004). Temperatures vary little between day and night but substantially between the seasons, with January the warmest

month averaging 1°C compared with winter temperatures of below -20°C. Blizzards are not common in the Vestfold Hills region due to the surrounding hills. This has led to the term *Riviera of the south,* often used to describe the Vestfold Hills.

It was around the Davis Base that samples of soil, snow, ice, rocks and organic material were collected and from which yeast isolates were obtained. These isolates formed the basis of the present research on the isolation and characterisation of yeasts from Antarctica.

Ultraviolet light

Ultraviolet (UV) light is a non-ionizing radiation in the electromagnetic spectrum and falls within the wavelengths 100 nm to 400 nm. UV light is classified into three regions according to wavelength: UVA (315 – 400 nm), UVB (280 – 315 nm) and UVC (100 nm – 280 nm). The Sun is the principal source of ultraviolet radiation (UVR) with a significant portion absorbed by the atmosphere before reaching the Earth's surface. UV radiation is a significant stress on all life forms. The thinning of the ozone layer over Antarctica due to photochemical catalysis by substances such as chlorofluorocarbons (CFCs) have been recorded for decades resulting in the annual *ozone hole* in spring. Since stratospheric ozone absorbs biologically damaging UVB, depletion has resulted in an increase in the amount of UVB radiation received at the Earth's surface (Malloy *et al.*, 1997).



http://www.srrb.noaa.gov/

Figure 1.6. Scheme showing penetration of the ozone layer by UVR

UVC, closest to the X-ray region, is essentially filtered in the stratosphere by ozone, preventing it reaching the Earth's surface. The solar UV that actually reaches the Earth is thereby primarily composed of UVA and UVB, also referred to as near and far ultraviolet respectively [Fig 1.6].

Free radical damage

Exposure to solar UVR is known to be associated with various skin cancers, cataracts of the lens as well as other eye diseases, photoaging of the skin and suppression of the immune

response (Beissert and Granstein, 1996; Young, 2004; Halliday, 2005). To elicit damage, a cellular response has to occur. The absorption of a single photon at the molecular level produces an excited state where an electron of the absorbing molecule is raised to a higher energy level. Reactive oxygen species (ROS) and reactive nitrogen species (RNS), also known as free radicals, are the primary products of UV exposure resulting in oxidative damage to lipids, proteins and DNA (Halliwell and Gutteridge, 1999). ROS and RNS are also generated during normal metabolic processes such as respiration and are efficiently removed by cellular antioxidant defense systems. However, an imbalance between the latter and the (over) production of ROS and RNS can lead to oxidative stress. Oxidative stress is now recognized as a key factor in regulating signal transduction and gene expression, programmed cell death as well as being associated with numerous metabolic disorders and disease states [Fig 1.7].

The hydroxyl radical, $HO^{\bullet}$, is one of the most reactive oxygen species. It can be formed by a number of reactions, one of which is UV light photolysis of H_2O_2 (reaction 1):

$$H_2O_2 + \text{UV light} \rightarrow 2HO^{\bullet} \qquad (1)$$

The hydroxyl radical can interact directly with an organic substrate (such as the nucleic acids that form DNA) by abstracting an electron (reaction 2). The resulting product is also a radical and can react in turn with ground-state oxygen to produce a peroxyl radical that is also highly reactive (reaction 3) resulting in a classic chain reaction.

$$HO^{\bullet} + R \rightarrow R^{\bullet} \rightarrow H_2O \qquad (2)$$

$$R^{\bullet} + {}^3O_2 \rightarrow ROO^{\bullet} \qquad (3)$$

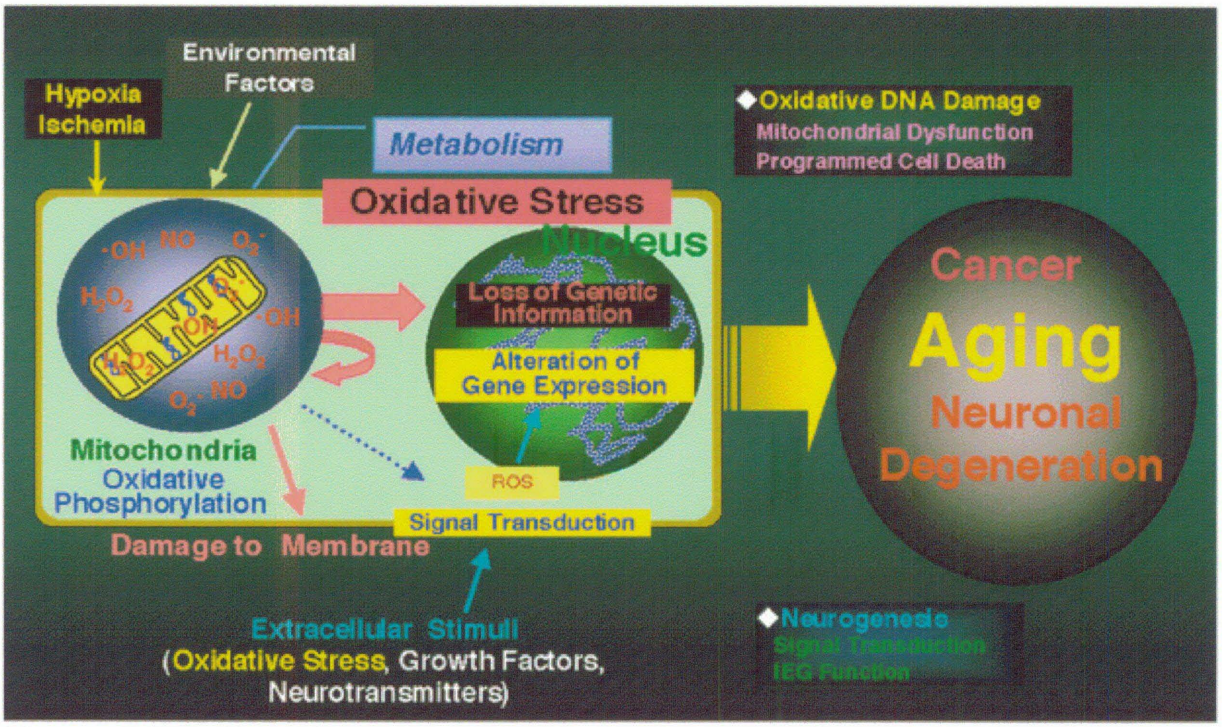


Figure 1.7. Schematic overview of cellular oxidative stress
http://www.med.kyushu-u.ac.jp/neuro/core/image/nakabe1.gif

This chain reaction results in oxidative damage of fatty acids and other lipids and, as such, has been implicated in a number of human disease states such as atherogenesis, carcinogenesis, as well as the ageing process (Halliwell and Gutteridge, 1999). Free radical attack at protein amino acid side chains occurs primarily by hydrogen abstraction resulting in protein oxidation. This protein oxidation can in turn cause modification of lipids, DNA and proteins resulting in a cascade of events causing further cellular damage.

Antioxidants

The antioxidant defense system is a highly complicated system that functions to protect cells against the potentially harmful effects of ROS. Antioxidants can be classified into three classes: antioxidants that prevent ROS formation; antioxidants that scavenge ROS; and antioxidants that repair the damage caused by ROS. The antioxidant defence system is composed of both enzymes and low molecular weight compounds.

The major enzymatic antioxidants include superoxide dismutase (SOD), glutathione peroxidase and catalase. SOD scavenges superoxide anions converting them to hydrogen

peroxide, which in turn is removed by catalase and glutathione peroxidase (Halliwell and Gutteridge, 1999).

The low molecular weight antioxidants comprise of a diverse range of compounds that protect cells from oxidative damage by direct and indirect interactions with ROS. The indirect interactions involve the removal of transition metals (e.g. Fe^{2+}) thus preventing their involvement as catalysts in the production of hydroxyl radicals. The direct acting antioxidants donate an electron to oxygen radicals thus quenching the radical. Direct acting antioxidants include reduced glutathione, carotenoids and vitamins C, D and E (Halliwell and Gutteridge, 1999).

Effect of UVR on Antarctic organisms

Depletion of stratospheric ozone during the austral spring results in an increase in the amount of UVR reaching Antarctic terrestrial and marine habitats. Marine phytoplankton have been shown to be negatively affected by solar UVB radiation by reduction of productivity, growth and survival rates (Hader, 2000) Shifts in spectrally dependent processes such as photoinhibition, photoreactivation, photoprotection and photosynthesis have also been reported (Smith *et al.*, 1992). Phytoplankton form the base of the aquatic food chain. Alterations in the size and composition of the phytoplankton communities directly affect food production from aquatic sources. Many unicellular marine organisms are highly sensitive to UV radiation. The delicate balance of ecosystems can be altered by the selection of UV-resistant species thereby threatening the growth and survival of other more UV-sensitive microorganisms (Hader *et al.*, 2003).

Studies of marine bacteria have shown interspecies variability to UVB sensitivity and repair capabilities (Arrieta *et al.*, 2000). Ocean dwelling microbes are exposed to UVB radiation levels inversely proportional to the depths at which they reside. UVB penetrates to significant depths of 20 – 30 m at intensities that cause measurable biological damage (Smith *et al.*, 1992; Malloy *et al.*, 1997).

Many organisms have evolved the ability to produce or accumulate photoprotective compounds such as UV-absorbing mycosporine-like amino acids (MAAs). These MAAs have

been identified in a variety of organisms including cyanobacteria, fungi and algae as well as a host of marine organisms such as corals, sea anemones and fish (Mason *et al.*, 1998; Newman *et al.*, 2000; Shick *et al.*, 2002; Volkmann *et al.*, 2002; Libkind *et al.*, 2004a). These compounds are thought to provide protection by acting as UV-absorbing compounds (max 309 – 360 nm) (Shick *et al.*, 2002). Studies of marine organisms of the Great Barrier Reef indicate a correlation between the exposure of organisms to solar or artificial UVR and their concentration of MAAs (Shick and Dunlap, 2002). The ability of these organisms to withstand long term UV exposure has led to the proposal that MAAs have a role as a natural UV sunscreen (Dunlap *et al.*, 1999). There are over twenty known MAAs with structural diversity resulting from substitutions of various amines, amino acids and amino alcohols at two positions on the MAA base structure. MAAs are products of the shikimic acid pathway and are closely related to the mycosporines found in fungi and, as such, are not expected to be found in animals that lack this biochemical pathway (Shick *et al.*, 2002). Production of MAAs as well as biochemically associated antioxidant defences have been reported in several yeast genera such as *Rhodotorula, Sporobolomyces, Cryptococcus* and *Phaffia* (Libkind *et al.*, 2004a). In higher animals, MAAs are present depending on diet and bacterial modification within the consumer (Dunlap and Schick, 1998). MAAs are found in marine organisms, including bacteria and yeasts, in a wide range of environmental habitats, from tropical reefs and lakes to the freezing waters of Antarctica. The UV-absorbing ability of MAAs in Antarctic marine organisms contributes to the protection of the marine food chain affected by high UVR.

Carotenoids and UV

Carotenoids are found in humans, plants, many species of fungi and certain groups of bacteria. They are lipophilic micronutrients and in humans are predominantly found in blood and tissues, including the skin and eye. Carotenoids consist of eight isoprenoid units arranged so that the isoprenoid units are reversed at the centre of the molecule [Fig 1.8]. Classed as hydrocarbons (carotenes) and their oxygenated derivatives (xanthophylls) they are found as components of the majority of natural pigments, the most abundant are β-carotene and xanthophylls (zeaxanthin, canthaxanthin and astaxanthin).

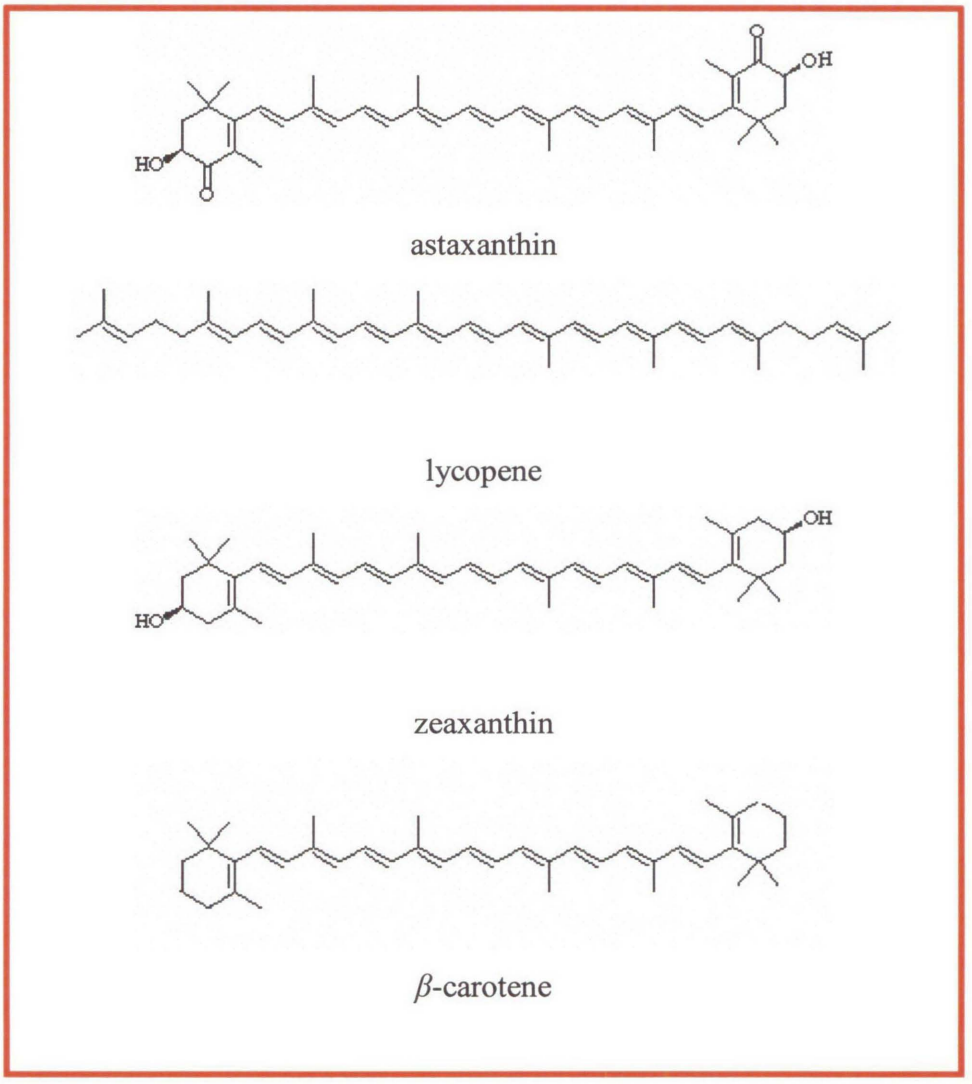


Figure 1.8. Chemical structures of astaxanthin, lycopene, zeaxanthin and β-carotene

Singlet oxygen and the peroxyl radicals are efficiently scavenged by carotenoids. Various pathways are operative when quenching singlet oxygen. In summary, singlet oxygen is deactivated by energy transfer from the excited state oxygen species to the carotenoid forming a triplet excited carotenoid. The excited carotenoid returns to the ground state through vibrational interactions with the solvent (Kennedy and Liebler, 1992). Carotenoids are lipophilic antioxidants found in lipoproteins such as low-density lipoproteins (LDL) and high-density lipoproteins (HDL).

UV exposure is believed to be responsible for numerous pathologies, such as skin erythema (Saliou *et al.*, 2001), systemic immunosuppression (Garssen *et al.*, 1998), carcinogenesis (Marks, 1999) and photoaging of the skin (Saliou *et al.*, 2001). UVB is primarily absorbed in the epimermis leading to an increase in cellular ROS and RNS resulting in oxidative damage to lipids, proteins and DNA (Saliou *et al.*, 2001). There have been numerous epidemiological studies supporting the theory that carotenoids provide moderate protection from UV-induced erythema (Stahl *et al.*, 2000; Stahl *et al.*, 2001). This protective role is attributed to the ability to scavenge ROS generated in photooxidative processes (Heinrich *et al.*, 2002). There is a large body of epidemiological evidence indicating an inverse relationship between carotenoid intake and cancer risk (Black, 2004), however, three β-carotene intervention trials : the Beta-Carotene and Retinol Efficacy Trial (CARET), Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study (ATBC) and the Physician's Health Study (PHS), have all reported negative effects of β-carotene supplementation on lung cancer risk in individuals already at risk, e.g. smokers (Patrick, 2000; Heinrich *et al.*, 2002; Bendich, 2004).

There is considerable focus on the natural production of carotenoids by microorganisms such as bacteria and yeast. In the case of yeasts, increased production of physiologically significant carotenoids (e.g. lutein, lycopene and α-carotene) can be achieved by exposing cells to exogenous stress factors such as UVR. *Rhodotorula glutinis*, *Sporidiobolus salmonicolor* and *Phaffia rhodozyma*, the so-called industrial red yeasts, are a rich source of astaxanthin. Carotenoid production is increased in these strains under various exogenous stress conditions, such as, osmotic stress, oxidative stress, heavy metals, UV-irradiation and ethanol stress either alone or in a range of combinations (Drabkova *et al.*, 2003; Koci *et al.*, 2003a; Koci *et al.*, 2003b; Pokorna *et al.*, 2003). Given the protective properties that carotenoids exhibit, it not unreasonable to hypothesis that they may serve as a mechanism of survival for yeasts found in extreme habitats such as Antarctica.

Heat shock response

All organisms have developed various complex intracellular defence strategies to combat stress. One such strategy is the selective expression of a set of proteins collectively known as the *heat shock proteins* (hsps). The synthesis of the hsps constitutes the universal *heat shock response* found in every organism from bacteria to humans (Morimoto *et al.*, 1990;

Watson, 1990). The heat shock response results in transcription of heat shock genes resulting in preferential synthesis of the hsps. Hsps may be classified into families and are named according to their molecular weights as estimated by 1D-SDS-PAGE. These groups are the large hsps consisting of a wide range of proteins of approximately 100-110 kDa, 82-90 kDa, 70 kDa family, 60 kDa family, 40-50 kDa, the small hsps approximately 15-30 kDa and ubiquitin, an 8.5 kDa ptotein (Morimoto *et al.*, 1990; Watson, 1990). Although first identified in *Drosophila* more than forty years ago (Ritossa, 1962), subsequent studies have shown that the heat shock response is universal among all organisms. Moreover, the hsps are highly conserved among widely divergent organisms. For example, hsp70 has approximately 50% of its sequence conserved between the bacterium *Escherichia coli* and human, with some domains as high as 96% (Schlesinger, 1990). Such high evolutionary conservation of the genes involved in the heat shock response suggests that this function may be essential for survival during hostile conditions (Schlesinger, 1990; Trautinger, 2001).

Many hsps are induced under altered environmental and physiological conditions, such as variation in temperature, environmental pollutants (e.g. metal), free radicals, alkylating agents and pathophysiological states such as fever, ageing, ischaemia and inflammation (Morimoto *et al.*, 1990). The conditions that induce hsps can be broadly classified into three groups, environmental, pathophysiological and physiological as indicated in Table 1.3. Heat shock proteins play a critical role in cellular recovery from stress by functioning as molecular chaperones. Molecular chaperones recognize nascent polypeptides, unstructured areas in proteins as well as exposed hydrophobic regions of amino acids. This allows molecular chaperones to protect stressed cells by holding or refolding denatured proteins as well as facilitating movement across membranes or through subcellular compartments (Morimoto *et al.*, 1990; Nollen and Morimoto, 2002; Calabrese *et al.*, 2003). Their role therefore, is to counteract the damaging effects of protein denaturation as a result of cellular stress by preventing or restoring proteins or by eliminating irreversibly damaged proteins.

Molecular chaperones are ubiquitous proteins and many are constitutively expressed under normal conditions, maintaining homeostasis by regulating protein folding of newly synthesized proteins and assisting in protein translocation across intracellular membranes. Under stressed conditions (e.g. heat shock) expression of many hsps are up-regulated including

members of the hsp100, hsp90, hsp70, hsp60, hsp40 as well as many of the small hsps (Nollen and Morimoto, 2002).

Table 1.3: Conditions that induce heat shock proteins

Category	Stressor
Environmental stressors	Heat
	Heavy metals
	Ultraviolet radiation
	Amino acid analogs
	Alcohols
	pH
	Insecticides and pesticides
	Antibiotics
	Salinity
Pathophysiological stressors	Viral infection
	Ischaemia
	Inflammation
	Oxidative stress
	Ageing
Physiological stressors	Cell cycle
	Growth factors and cytokines
	Cell differentiation

Adapted from Lahav *et al.* (2003); Macario and Conway de Macario (2000); Morimoto *et al.* (1990); Simon *et al.* (1995); Trautinger (2001).

Exposure of cells to a mild stress results in an increased expression of hsps and is followed by a period of resistance to further stress challenges that may otherwise have led to serious cell injury or death, thus allowing cells to adapt to environmental changes that would otherwise be lethal (Watson, 1990). This phenomenon is known as acquired tolerance and it was first described over two decades ago in *Saccharomyces cerevisiae* for thermotolerance (McAlister and Finkelstein, 1980) and ethanol tolerance (Watson and Cavicchioli, 1983). It has been shown that induction of hsps is associated with acquired tolerance to a wide range of stressors, other than heat and ethanol. Hence, the term stress proteins have been applied to the hsps (Watson, 1990). It is important to note that acquired stress tolerance is a transient state and cells characteristically lose stress tolerance over a recovery period. The kinetics of loss of acquired tolerance is a function of the stressor, stress conditions and cell type (Watson, 1990).

Heat shock response to UV

Ultraviolet radiation is one of the most harmful environmental factors on human health and is responsible for several biological effects on human skin including, erythema, carcinogenesis and photoageing. The increase in ultraviolet influx caused by ozone depletion is of concern as the majority of the acutely sensitive Australian population engages in extensive UVR exposure. UVR exposure has been shown to increase levels of ROS and RNS in cells resulting in oxidative damage to lipids, proteins and DNA (Parsons, 1997; Jones *et al.*, 1999).The cellular response to UVR is dependant upon tissue type and wavelength. UVC is strongly absorbed by DNA due to its shorter wavelength, which can lead to dimerization of adjacent pyrimidines on the same DNA strand resulting in a rapid induction of DNA repair systems which then leads to enhanced spontaneous and induced mutation frequencies (Tyrell, 1996) thereby allowing time for repair of DNA damage. The p53 protein can also induce apoptosis and this apoptotic pathway appears to be involved in suppression of tumour growth.

UVB radiation, like UVC, is absorbed by DNA molecules and as such activates a similar set of genes to UVC. The short wavelengths of UVB (as compared with UVA) penetrate sufficiently into the skin to cause severe damage in both the dermis and epidermis layers. There have been a number of reports on the induction of hsps by UVR, however, conflicting data indicate that the experimental conditions as well as the cell type have significant effects upon the results (Peng *et al.*, 2000). Exposure to a mild heat shock resulting in increased expression of the hsp70 family and protects against UVB exposure in both murine and human keratinocytes (Simon *et al.*, 1995; Trautinger *et al.*, 1995) . Constitutive expression of hsp72 in human epidermal cells has been shown to have a protective effect against UVB and overexpression of hsp72 is involved in heat-induced UVB resistance (Trautinger *et al.*, 1995). These researchers were able to inhibit the constitutive as well as the stress induced expression of hsp72 in an epidermal cell line resulting in the enhanced damaging effect of UVB. The longer wavelength of UVA radiation indirectly induces DNA damage by photochemical reactions generating free radicals and other active species such as singlet oxygen (Tyrell, 1996). UVA leads to increased expression of a diverse range of genes, for example; heme oxygenase 1 (HO-1), protein phosphatase (CL100) and collagenase (Tyrell, 1996).

Thesis Aims

Yeasts isolated from extreme environments such as Antarctica, offer a unique opportunity to exploit characteristics not found in other microorganisms. The focus of this study was sixty yeast samples originating from the Vestfold Hills area located near the Australian Davis Base, Antarctica. The Vestfold Hills region is a particularly rich area in terms of ecological diversity. Consisting of both salt and fresh water lakes, the area is protected from the strong winds so common in Antarctica by the surrounding hills. Previous studies from this laboratory of yeasts isolated from this region initiated a thorough literature review to examine yeast biodiversity in Antarctica. Although there have been a number of biodiversity studies in regards to Antarctic bacteria, studies focusing on yeasts are lacking.

Samples were obtained in the austral summers of 1994/1995 and 1997/1998 and included soil, organic material as well as snow and ice. Yeasts were isolated from these samples and classified according to their maximum growth temperatures (Thomas-Hall, 1997; Guffogg, 2001). One-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (1D-SDS-PAGE) of whole cell proteins was adopted as an initial screen to group isolates with similar growth and morphological traits. Isolates with identical or very similar protein profiles were considered to be conspecific and, as such, a representative isolate was chosen for subsequent analyses. This screening process reduced the study group from sixty to forty-four isolates. Functional studies of these extremophiles using a combination of classical and molecular techniques provided a correlation analysis that was applied to the placement of new species against positions of existing species. To date, 3 novel species have been identified as well as the characterisation of a considerable number of established yeast species, not previously isolated from Antarctica.

The introduction of molecular techniques has resulted in an escalation in the identification of new yeast species due to the alacrity and accuracy with which these techniques can be performed. These techniques include the sequencing of the D1/D2 region of the ribosomal DNA of yeasts and, coupled with the very limited studies on the biodiversity of Antarctic yeasts were the focus of much of the aims of this thesis.

During the course of this work, opportunity arose through collaboration with the Australian Institute of Marine Science, to analyse the coenzyme Q (CoQ, ubiquinone) systems of the Antarctic yeasts not only from the viewpoint of taxonomy, but also as a sensitive measure of cellular redox potential and oxidative stress under UVA radiation. A highly pigmented yeast, subsequently identified as *Rhodotorula mucilaginosa*, was shown to have an unusual ubiquinol:ubiquinone ($CoQH_2$:CoQ) ratio indicative of an efficient antioxidant capability that was not seen in the majority of the other Antarctic yeasts analysed. This result prompted further investigation of the ability of this yeast to circumvent environmental stressors such as heat, UVA and UVB radiation. In particular, a series of experiments were designed to investigate the stress response in this yeast in regards to hsp expression. Furthermore, as experimental work progressed, it was decided to conduct a small-scale clinical trial on humans to examine the effects of coenzyme Q, a commonly used dietary supplement, on stress protein synthesis and a number of parameters associated with antioxidant and oxidative stress status.

In summary, the aims of the thesis were to screen samples of Antarctic soil and related material for yeast biodiversity using:

a) 1D-SDS-PAGE of cell protein extracts as an initial screen to group yeast isolates with similar growth and morphological traits
b) Sequencing of the D1/D2 region of the large ribosomal DNA unit
c) Applying classical biochemical and taxonomic analyses
d) Determining the nature of the coenzyme Q systems in the isolates
e) Analysing the novel response to heat and UV-radiation stress in a highly pigmented Antarctic yeast, *Rhodotorula mucilaginosa.*