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PRACTICAL MYCOLOGY FOR INDUSTRIAL BIOTECHNOLOGISTS

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Preface

Fungi, the non-chlorophyllous, heterotrophic eukaryotes of the microbial world have been the subject of interest for academic studies, as well as, practical applications. Earlier mycological research focused on the destructive aspects of fungi as noxious plant and animal pathogens, and the studies on their life-cycles were primarily oriented for combating diseases and controlling infections. The discovery of penicillin, as well as, the application of saprophytic fungi like aspergilli in the manufacture of industrially useful products like alpha amylase and citric acid has provided considerable impetus to the study of saprophytic fungal genera and other species occurring widespread in nature which contribute significantly to recycling of organic matter, through breakdown of complex organic substrates of plant and animal origin. Further, bioactive fungal metabolites and industrially useful enzymes of fungal origin have firmly established their utility in the rapidly advancing area of fermentation technology. Currently search for novel fungal taxa and their application for discovering newer metabolites of value have gained considerable momentum. This in turn has brought into focus the need for a better understanding of the ecology and distribution of the fungi in the natural environment, their *in vitro* culture and effective long term conservation in germplasm banks and, above all, a better insight into fungal taxonomy and classification. Morphogenesis of fungi under fermentation conditions and the regulation of environmental parameters to optimize yield and productivity of metabolites has also acquired considerable importance. Mutation for yield improvement through classical techniques as well as molecular biology and genetic engineering approaches have been successfully carried out and heterologous gene expression to produce mammalian proteins like chymosin in fungal systems is a reality.

My initiative to write this book originated from the realisation that fungi are becoming more and more important in applied microbiology and biotechnology. And there have been very little effort is to comprehensively compile information related to the biology of fungi and their biotechnological aspects. Such a book would be very useful to students of industrial biotechnology as well as research and development personnel involved in developing processes for fungal based bio-products. In the book, focus is specially laid on the aspects of ecology, distribution and biodiversity of fungi together with selective techniques for isolation and cultivation of fungi in artificial culture and their long-term conservation to enable biotechnological explorations.

In undertaking to present the details from such a wide range of topics related to fungi, I am certain that there will be several shortcomings and deficiencies readily apparent to those with high level expertise in specific areas. It is perhaps best to recognize the useful data that this compilation is likely to offer to the practical myclogists and biotechnologists and thereby catalyse their interest and enthusiasm to undertake investigations on diverse fungal systems leading to newer innovations and discovery of novel bioactive molecules.

M C Srinivasan

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M C Srinivasan

Contents

CHAPTER 1

The Mycological Kingdom

Ecology and Distribution, Biodiversity, Classification and Taxonomy of Fungi

History of Mycology

Mycology—the study of fungi—has been an important development in human civilisation. Records of the utilisation of macrofungi and macrolichens as food by man date back to early Greek and Roman classics. Systematic investigations of fungi perhaps did not take place until the eighteenth century and the foundations of this science were firmly laid by those pioneering researchers, who investigated the ravages of fungal diseases on plants. Progress in mycology closely followed the advances in plant pathology and the measures adopted to control the diseases on plants. Macrofungi like the mushrooms which are visible and readily collected from the field received considerable attention both in terms of their classification and identification and also their recognition as edible and poisonous varieties. Ergot poisoning from contaminated grain lots was another early milestone discovery on the hazards of fungal infection on human health. In the orient, fermented foods involving saprophytic moulds were known for long but their mycological investigations have been relatively recent. Mycology, over the years has been in the domain of botanists although the fungi, as we understand presently are more closely related to the animal kingdom!

Systematic mycology including naming of new genera and species and their classification has been an intensive activity of mycologists in different countries and the contributions of Indian mycologists for enumerating the fungal flora of the Indian subcontinent has been very significant. The "*Fungi of India*" was first published by **E.J. Butler** and has been subsequently revised to include several new records of fungi, many of them new to science. These include both pathogenic and saprophytic fungi from various substrates including soil. The recognition of soil as a natural reservoir for a variety of fungi has led to their isolation and study in pure cultures and in recent years several genera and species of soil fungi have proved to be useful resource pools for developing processes for the manufacture of a variety of bioactive molecules and industrially useful enzymes.

Recent advances in applied microbiology and fermentation technology have established the potential of fungi as very valuable sources for useful metabolites and presently there is a great deal of interest in exploring the saprophytic species for diverse metabolites. In order to undertake such studies on mycological exploration, it is obvious that a sound knowledge on the ecology and distribution of fungi in the natural environment, their biodiversity and also their classification and taxonomy would be most desirable. In this chapter, these three aspects are discussed in detail.

Ecology and Distribution

Ecology is the study of the interactions of organisms and their environment. Biological behaviour as expressed in their phenotypes is the result of genetic expression directly related to and regulated by proteins which are synthesised and expressed in response to internal and external environmental controls. Ability to adapt to an environment is inherited but the phenotypic state resulting from the adaptation is directed towards survival in the ecosystem and comprises long term adaptation (evolutionary adaptation) and short term variations

which are essentially physiological adaptations. Evolutionary adaptation is a result of mutational changes in the genotype and selection of the most favoured and suited for survival in the particular environment. Physiological adaptation on the other hand represents short term changes taking place in populations of organisms in response to environmental variations.

In the natural ecosystem, fungi occupy a wide range of habitats and ecological niches. Pathogenic fungi, causing disease on plants and animals and humans, have a saprophytic capability of survival in the environment, often tiding over the unfavourable season in the form of dormant spores or thick walled chlamydospores often colonising organic debris particles. Plant pathogenic fungi produce spores with a long period of dormancy prior to germination, thereby ensuring survival during the off season when the host plants are not available in the field for infection (e.g. oospores of downy mildew fungi like *Sclerospora graminicola*, rust (*Puccinia graminis*) teleutospores, smut (*Tilletia foetida*) chlamydospores etc.). In the terrestrial ecosystem, saprobic fungi grow in soil, plant litter, rotten wood or other organic debris. Symbiotic fungi form ectomycorrhiza on a variety of vascular plants or occur endophytic within the host tissue as endosymbionts. Thermophilic fungi represent a special group which constitute a major component of the microflora developing in heaped masses of plant materials, agriculture and forest product piles and other self-heating accumulations of organic matter such as compost. They also occur in animal dung, bird's nests and tobacco products like snuff. The warm, humid and aerobic environment provides the basic conditions for their extensive development in these habitats.

Fungi were regarded as totally aerobic, requiring oxygen for growth (yeasts and some filamentous fungi like *Mucor* could carry out fermentation of sugars in the absence of air to form ethyl alcohol). This concept was given up with the recognition that among the microflora participating in the digestive processes in the anaerobic environment of the rumen of herbivores, were present truly anaerobic flagellated fungi (**Orpin.** *J. Gen. Microbiol*. 99:107–17, 1977). Extensive studies in subsequent years led to discovery of several genera and

species. In their thallus form and production of flagellated cells, these fungi have been classified under Chytridiomycetes as a separate order designated Neocallimastigales. It has been suggested that because these fungi produce rhizoids and can effectively penetrate fibrous plant material, they may have a role to play in the initial colonisation and degradation of structural carbohydrates altering the physical structure of the fibre and making it less refractory for the reactions of other microbes in the rumen. In vitro cultural studies have demonstrated that these fungi make significant contributions to the solubilisation of plant cell wall material and can participate in the lignocellulose digestion for the host herbivorous animal. A detailed review of these rumen anaerobic Chytridiomycetes has been published (**Trinci** et al. *Mycological Res.* 98:129–52, 1994). It is significant to note that these fungi represent the first record of true anaerobic life forms among eukaryotes.

Apart from the terrestrial habitats, fungi are also widespread in aquatic environments and often comprise forms uniquely adapted to grow and survive in these environments. Fresh water fungi comprise aquatic phycomycetes (both unicellular and filamentous forms) producing flagellated zoospores in sporangia (asexual reproduction) and flagellated male gametes (antherozooids) which swim towards female gametangia by chemotaxis and hormonal influence to fertilise the non-motile female gametes. The other major group of fresh water fungi are the aquatic hyphomycetes, often referred to as "Ingoldian" fungi (in honour of the famous British mycologist, **C.T. Ingold**, who first identified and extensively studied them). These are recovered from foam samples in clear aquatic streams or from decomposing plant tissues underneath clear water surfaces. These are characterised by spores with one or more radiating arms or appendages, which help in their buoyancy and ability to float in the aquatic environment besides facilitation of anchorage to substrates and their colonization. A survey of tropical fresh water hyphomycetes with excellent illustrations of diverse forms has been published (**Marvanova.** in *Tropical Mycology* (edited by **K.K. Janardhanan** et al. Oxford & IBH Publishers Co., New Delhi p.169–226, 1997).

Marine fungi comprise saprobic forms present in the open ocean waters (pelagic) and in the bottom (benthic) zones. However the majority of studies on marine fungi have related to forms occurring in various types of submerged materials in waters and sediments nearest to land—the neritic and littoral zones. Little knowledge exists of fungi present in deep waters and associated sediments. Submerged wood in marine ecosystems has received considerable attention for mycological studies and predominantly several unique ascomycetes with often appendaged spores and dematiaceous hyphomycetes have been identified. Apart from submerged wood, calcareous deposits as well as accretions of marine animals too harbour unique fungal flora. Intertidal and salt marsh flowering plants like *Spartina* and *Juncus* as well as invertebrate animals like coelenterates and sponges and also various groups of macroalgae harbour their own fungal associates which are unique and distinctive. One group of marine fungi, which have received considerable attention in recent years is the thraustochytrids. Species of *Thraustochytrium*, *Schizochytrium* and related forms have received attention from biotechnologists for their high content of Polyunsaturated Fatty Acids (PUFA) especially Docosahexaenoic acid (DHA). Members of the genus *Thraustochytrium* are characterised by monocentric thalli that are attached to their substrate by branching epibiotic or endobiotic rhizoid-like extensions termed the ectoplasmic net. Zoospores are formed when the vegetative thallus transforms into a zoosporangium after which the zoospores are released as non-flagellate angular bodies. These after a period of rest get transformed into motile pyriform spores with two flagella.

Other ecological systems with their individual fungal flora include animal dung, seeds, plant litter and decomposing wood. Soil as a reservoir of diverse mycoflora harbours these groups as a result of getting incorporated into itself the products of decomposition of these natural substrates following microbial degradation and recycling of organic matter. Keratinophilic and chitinophilic fungi widely distributed in soil samples are examples of specialised groups that are capable of colonising and degrading keratin and chitin which are not readily degraded by most fungi. Dermatophytes

and some others are the only fungi that are capable of breaking down keratin and it is obvious that fungi causing skin infections on man and animals are widely distributed in soil samples around the world, and more so in tropical countries where the climatic conditions are more favourable for their development.

Dung inhabitating fungi are collectively termed as coprophiles and these comprise predominantly Zygomycetous fungi, Ascomycetes and Hyphomycetes. Members of Mucorales as well as ascomycetous genera like *Chaetomium* and *Sordaria* readily develop on dung specimens incubated in moist chambers. The Mucoralean fungus *Pilobolus* is an example of a coprophilous fungus which has an obligate requirement for a growth factor extracted from dung for its growth. This factor designated coprogen, (**Hesseneltine** et al. *Mycologia* 45:7–19, 1953) has been chemically characterised (**Pidacks** et al. *J Amer Chem Soc*, 75:6064–65, 1953).

Seeds harbour a rich and varied mycoflora through fungal invasion of the seed during different stages of development as well as during post-harvest storage. Seed borne infection of phytopathogenic fungi and seed transmission of disease to newer locations is a serious matter necessitating stern quarantine measures. This has led to advances in seed pathology and seed science technology for devising reliable methods for detection of seed-borne phytopathogenic fungi and also for devising effective control measures to contain disease outbreaks originating from seed-borne inocula. Seed-borne fungi are also the causal agents of toxic metabolites harmful to humans and animals who consume them. The best known example and one which has been extensively investigated is the aflatoxins, which are mycotoxins produced in peanuts by *Aspergillus flavus*. These toxins are responsible for producing liver carcinogenesis and similar symptoms leading to fatal situations. When seed samples are incubated on moist blotters in petridishes, several hyphomycetous fungi are readily encountered among which *Alternaria*, *Aspergillus*, *Cladosporium, Curvularia, Drechslera* and *Fusarium* are the most frequent. Serious fungal pathogens of plants transmitted through seeds include

Pyricularia oryzae causing blast disease of rice and smut diseases such as loose smut of wheat caused by *Ustilago tritici*. Deterioration of seed quality after harvest and during storage is caused by xerotolerant fungi, notably species of *Aspergillus* such as *A. glaucus, A. restrictus* etc. These fungi possess unique ability to tolerate and grow under low water activity (*a*

Osmotolerant and halotolerant fungi are isolated most frequently from sugared or salted food products, from concentrated fruit juices and from damp sugar and salt. Ability to develop in high sugar and salt concentration has been acquired by these fungi through adaptation of their enzymes to function under high osmotic pressure and under their optimum pH and temperature. Notable examples of halotolerant fungi include species of *oospora* isolated from salted fish which grew best in media with 15–20% salt and failed to grow in media with less than 5% sodium chloride. It also grew well in 65–70% sucrose solution (**Malevich.** *Mikrobiologiya* 5:813–17, 1936). *Aspergillus halophilicus* having *Eurotium* perfect state was isolated from wheat seeds on media with either 15% sodium chloride or 70% sucrose and this fungus showed very poor growth in media containing less than 10% sodium chloride or 50% sucrose (**Christensen** et al. *Mycologia* 51:636–40, 1959).

Litter and Wood Decomposing Fungi

Plant litter is a major contributor to organic matter recycling and under natural conditions is a seat of diverse microbial activity in which fungi play a major role. The litter in undisturbed forest floors comprising multiple layers of fallen leaves and barks and twigs constitute the detritus which is decomposed by the activity of several microbes as well as other life forms such as protozoa, insects and even small animals like rodents. Forest litter is also enriched by bird and animal droppings, feathers, hair etc. and can be expected to provide ecological niches for specialised groups of fungi such as the coprophiles, keratinophiles and chitinophiles. Decomposing proteinaceous residues and the consequent release of ammonia would also provide a desirable environment for the multiplication of alkalitolerant microbes and possibly even obligate alkalophiles.

Hayes (Science Progress 66:25–42, 1979) in his review on the microbiology of plant litter decomposition pointed out that

it has basic implications for the cycling of carbon and nitrogen and for the supply of macro and micronutrient elements for green plants. "Incredibly little is known of the microbiological processes in humification or of the role played by any particular fungi" and he concluded that "the greatest single barrier to further advances in this field has been the failure to attract biochemists and enzymologists who alone are likely to unravel the intractable problems of the process." More than two decades after this analysis, the situation has not improved and unfortunately few systematic studies have been carried out to get meaningful data which would help in understanding litter decomposition better from the biological as well as the biochemical angle. The complexity of plant litter decomposition is a challenging one since litter composition is highly variable depending on the major contributing plant species. While cellulose, hemicellulose and lignin in varying proportions would be the common entities, various plant extractives and chemical compounds exclusive to specific ecosystems would exert profound influence on the component microflora and the pattern of degradation. Relatively few studies, especially isolation and in vitro cultural studies on litter fungi have been published. This is surprising since the biodiversity of litter microbes and their respective roles in the degradation process would provide novel gene pools for enzymes involved in the degradation of a wide range of natural polymers including carbohydrates and proteins.

Some broad generalisations on the succession of mycoflora acting in synergy for the decomposition of plant litter have been attempted. It has been observed that the main phase of decomposer activity is effected by a group of common saprophytic fungi such as *Cladosporium herbarum, Alternaria tenuis, Botrytis cinerea,* etc. Initial colonisation is perhaps initiated by species which can utilise the small quantities of soluble carbohydrates available. These microbes with xylanolytic and cellulolytic potential appear to play a significant role in solubilising the plant polymers to produce sugars on which the above mentioned and related species of fungi can rapidly grow. During further succession on the decomposing litter, Ascomycetes, Basidiomycetes and even

Zygomycetes such as *Absidia*, *Mucor* and *Zygorrhynchus* can occur as part of the saprophytic mycoflora. Species of the saprophytic Entomophthorales belonging to *Conidiobolus* and *Basidiobolus* have been discovered regularly in association with plant litter in an advanced state of decomposition in contact with moist ground.

Decomposition of woody substrates is primarily due to the action of fungi belonging to basidiomycetes and these often form their characteristic fructifications from the dead woody substrates under favourable conditions in the natural environment. The polypores are widespread forming their fructifications popularly referred to as the bracket fungi. The "brown rot" basidiomycetes have the ability to enzymatically degrade hemicellulose and cellulose, while the "white rot" basidiomycetes can in addition also degrade lignin. Wood decomposition is however a slow process in the natural environment. Many of the wood degrading fungal cultures are biotechnolgically significant as sources of valuable enzymes, finding application in wood pulp biotechnology. A white rot basidiomycete *Phanerochaete chrysosporium* (*Sporotrichum pulverulentum*) has been the subject of extensive research studies for its utilisation in biopulping operations in the paper industry.

Recent interest has focussed on the study of endophytic and bark associated fungi from medicinal plants. One of the most significant discoveries in this area has been the isolation of an endophytic fungus, *Taxomyces andreanae* from the bark of Pacific yew (*Taxus brevifolia*) which has been shown to produce Taxol, the antitumor diterpenoid produced by the Taxus plant (**Stierle** et al. *Science* 260: 214–16, 1993). The concept has been gaining ground since this discovery that the endophytic fungi associated with medicinal plants may have the potential to acquire the capability to produce the valuable metabolite of medicinal value from the parent plant and such fungi could become independent alternative sources for these metabolites which can be produced by fermentation. This opens a tremendous new area for research and development, particularly for screening the innumerable medicinal plants of indigenous origin for endophytes and evaluating them for

their potential to manufacture the valuable compounds of plant origin. A recent report from China has claimed success in producing the anticancer drug Vinblastin from *Vinca rosea* by fermentation employing an endophytic isolate of *Alternaria* from the periwinkle plant (*Chem. Abst.* 129:172900-s, 1998).

Biodiversity

Recognition of fungal biodiversity entails an appreciation of the variations in morphology that different taxa exhibit and their physiological differences which enable them to colonise and survive under different nutritional and environmental conditions. Thus studies on biodiversity related to morphological, physiological and ecological aspects can get integrated to provide a composite picture of biodiversity of any group of living organisms. In the case of fungi, morphology of spore forms as well as the vegetative thallus play a very important role in recognising the biodiversity. It is significant to note that recognition and classification of diverse fungi in the classical approaches to taxonomy has been entirely morphological.

Morphologically, fungi vary from microscopic unicellular forms to diverse macrofungi such as mushrooms, polypores and morels which are readily recognised in the field and identified by their morphological features. These belong to the Ascomycetes or Basidiomycetes. The lichens represent another interesting group of macrofungi growing under xerophilic situations and comprise symbiotic association between a mycobiont (a fungal component classified to belong to the Ascomycetes in most cases) and a photobiont (which is a green alga or a photosynthetic cyanobacterium).

Biodiversity, the extent of biological variation on earth, has come to the fore as a key issue in science and politics during the 1990s. First used as Bio Diversity in the title of a scientific meeting in Washington DC in 1986, the term has been rapidly adopted and contracted as biotic diversity and biological diversity. Recent interest in conservation of biological

diversity has been associated with destruction of forests, global warming and the need for conservation of naturally occurring genetic resources preventing their endangerment and consequent extinction. The most useful definition of biodiversity is that given by the International Union for Conservation of Nature and Natural Resources stating that biodiversity encompasses all life forms, ecosystems and ecological processes, and acknowledges the hierarchy at the genetic, taxon and ecosystem levels. In the words of the famous American naturalist, **Edward Wilson**, "Biodiversity is our planet's greatest but least developed resource for biotechnological innovation."

Most texts and reports on biodiversity lack any substantiative content on fungi or indeed in many cases on any groups of microorganisms. The scant attention paid to fungi in these debates on biodiversity is in part due to the lack of awareness among biologists of their significance in evolution, ecosystem functioning and human welfare. The kingdom of fungi encompasses a tremendously diverse and enormously versatile range of organisms gifted with the ability to utilise and recycle a large variety of organic and inorganic compounds occurring in the natural environment. Among the examples of extreme abilities for biodegradation, mention may be made of *Phanerochaete chrysosporium*, one of the key organisms in lignin breakdown can degrade persistent pollutants like Polychlorinated Biphenyls (PCB) and hold potential for cleaning up the environment of these toxic residues. This and several other fungi appear to be promising candidates for degrading toxic pesticides and other xenobiotic materials leading to cost effective removal of such substances from the environment by a process now gaining importance and termed bioremediation.

The tropical forests which occupy only 6% of the earth's land surface harbour more than 50% of living species and it is assumed that the fungal distribution also would follow this picture from general biological rules of food webs and interactions among organisms. The subject of biological diversity of tropical fungi is vast and the task of its evaluation

immense, especially since comprehensive data is presently lacking.

Hawksworth (*Mycological Res.* 95:641–55, 1991) estimated the total number of fungi to be 1.5 million of which around 69,000 species are currently known. The tropical regions would be expected to be the richest sources of new species and little explored habitats would be a major source for novel fungi. Examples include aquatic hyphomycetes, lichenicolous fungi, fungi on bryophytes, fungicolous mycoparasites, fungi on and in insects and other arthropods, nematode capturing fungi, fungi on native wild vascular plants and endophytic fungi forming mutualistic associations with the aerial parts of vascular plants. For instance anaerobic fungi living inside the rumen of herbivorous animals and species living inside rock surfaces in Antarctic represent highly specialised habitats. High specificity of distribution among fungi have been noticed in focussed studies undertaken to explore fungal populations. For example *Lantana camara* is known to support 55 fungi, 28 of which are apparently restricted to that host. Likewise 58 out of 87 fungi observed to grow on the lichen geuns *Peltigera* were specific to that species only. In a study of fungi associated with the Australian moss genus *Dawsonia*, twenty one Ascomycetes new to science were discovered (**Hawksworth.** *Mycologist* 11:18–22, 1997). These examples bear testimony to the fact that the present state of our knowledge related to fungal biodiversity in diverse habitats under the tropical ecosystem is in its infancy and there is an urgent need for undertaking systematic exploration for enumeration and identification of the vast fungal biodiversity which has so far remained untapped for biotechnology exploration.

To make an inventory of the fungal taxa in a defined area, the complete range of organic substrates must be sampled in all stages of development and decay over a period of time. **Russman** (A Strategy for all—taxa inventory of fungal diversity in *Biodiversity of terrestrial ecosystem* ed. **C.I. Peng**, **G.M. Chou**, 169–94, 1999) has considered that a mycological team of at least four experts are needed for field work with back up personnel in the laboratory isolating from these substrates. These include (1) a macrofungi specialist for

polypores, agarics etc.; (2) two microfungi experts to collect in specialised habitats above ground ranging from living leaves in the canopy to litter, twigs and deadwood. (3) a specialist for making isolations from plant parts, soil, roots and water. For a true and complete inventory of the fungal taxa an expert on fastidious fungi is necessary for isolation from anaerobic and extreme environments. Specialised techniques such as particle filtration and various selective media can be used to maximise the diversity of species isolated from particular substrates. As early as 1820, the famous mycologist **Elias Magnus Fries** had forecast that the fungi would prove to be the insects of the botanical world to imply their abundance and diversity in the natural environment. It would be obvious that the numbers of new fungi discovered in the tropics will be directly proportional to the time spent in intensive exploration of various habitats. Rare species or those requiring special methods or skills to be detected are less likely to have been discovered earlier and hence would be new and novel. Apart from innovative techniques for fungal exploration, high level expertise in fungal taxonomy would be an essential requirement for recognising fungal diversity. Unfortunately at present, the number of classical taxonomists with specialist knowledge in the taxonomy of specific groups of fungi is on the decline, particularly in the biodiversity-rich regions of the developing world. Necessary build up of expertise in the taxonomy and classification of fungi, both classical and molecular would be necessary before biodiversity prospecting for biotechnology involving fungal systems would become meaningful and beneficial.

How would fungal biodiversity be relevant to biotechnology applications? Microbial natural product screening for new drugs and biomolecules has been the backbone of the pharmaceutical and agrochemical industry for over fifty years. The era of antibiotic discovery has led to target-directed screens based on disease models in the search for low molecular weight molecules with receptor activity. The search for novel molecules is dependant on microbial biodiversity. Biodiversity, ecological niche exploitation and their abundance in association with other organisms characterise

the fungi, whose biotechnology potential is just beginning to be appreciated in good measure. The rapidity of growth and ease of cultivation in vitro have led to increased focus on the heavy sporulating hyphomycetes for a large majority of biotechnology explorations. *Penicillium chrysogenum*, the source of the life saving drug penicillin and *Aspergillus terreus* the source of hypocholestemic agent Lovastatin belong to the Hyphomycetes. Several compounds of pharmacological value have been identified in natural collections of mushrooms and other basidiomycetous fleshy fungi. Many of them are not produced in fermentations based on the mycelial cultures thereby posing problems in scale up for mass production. However several pharmacologically active secondary metabolites have been identified from vegetative mycelial cultures of Basidiomycetes and represent potential sources for novel useful biomolecules (**Anke** et al. J. Antibiotics 33:463–467, 1980).

Classification and Taxonomy

The early events related to the origin and evolution of fungi are not clearly understood as they are impossible to be established from fossil records. Probably the key events in fungal evolution took place in early Palaeozic or Precambrian. While classical biologists are familiar with the grouping of living forms generally under plants and animals, recent concepts have recognised the true fungi ("Mycota") as a separate kingdom. **Margulis** (*Biosystems* 27:39–51, 1992) pointed out that the field of systematic biology has been reorganised with logical technical definitions for each of the three major kingdoms of Eukaryotes (Mycota—true fungi, Plantae—Bryophytes and Tracheophytes and Animalia). **Wainwright** et al. (*Science* 260:340–42, 1993) analysed small subunit ribosomal RNA sequences and deduced that animals and fungi shared a unique evolutionary history—their last common ancestor being a flagellated protist similar to present-day choanociliates. **Baldauf** and **Palmer** (PNAS 90: 11558–62. 1993) constructed phylogenetic trees based on the

sequencing of four proteins, viz. alpha tubulin, beta tubulin, actin and elongation factor 1-alpha and showed that animals and fungi were related together as a monophyletic group while plants and a range of protists were unrelated to the fungi. It is obvious that animals and fungi are sister groups while plants constitute an independent evolutionary lineage. These concepts can be regarded as extensions to the classification that was proposed in 1969 by **Whittaker** (*Science* 163:150–60, 1969) where the biological kingdom was divided into five groups, viz. Monera (Prokaryotes: Bacteria and blue green alga), Protista (unicellular eukaryotic organisms), Plantae (multicellular green plants and higher algae), Fungi (Multinucleate higher fungi) and Animalia (Multicellular animals). In this classification, which was adopted in the seventh edition of the Dictionary of Fungi, the fungi were accepted as a single kingdom (monophyletic group). However with recent advances in ultrastructural, biochemical and especially molecular biological data, the treatment of fungi as a composite single group has become increasingly untenable. Fungi, defined broadly as "organisms studied by mycologists" are now established as polyphyletic (i.e. with different phylogenies) and have been referred to in at least three different kingdoms.

The three kingdoms of fungi accepted in the eighth edition of the Dictionary of Fungi (1995) are CHROMISTA, FUNGI and PROTOZOA. The Chromista are unicellular, filamentous or colonial eukaryotes which are primarily phototrophic and with cellulosic cell walls lacking chitin and β-glucan. Three fungal phyla recognised under Chromista are the Hyphochytriomycota, Labyrinthulomycota and Oomycota. The Hyphochytriomycota comprise saprobes on plant and insect debris or parasitic on algae and fungi, which are aquatic reproducing by zoospores with one anterior flagellum. The Labyrinthulomycota consist of two orders the Labyrinthulales and Thraustochytriales. They occur in fresh water as well as marine situations associated with higher plants or algal chromists. The third order Oomycota are widespread in aquatic as well as terrestrial habitats and include several economically important plant pathogenic fungi. The vegetative thallus is unicellular or mycelial, the hyphae being aseptate and coenocytic. The

zoospores are biflagellate with one flagellum being of the "tinsel" type having two rows of mastigosomes (hair-like structures covering the surface) and the other of the "whiplash" type (with smooth surface or covered by fine flexuous hairs). The cell walls contain glucan—cellulose and rarely with small amounts of chitin. The Oomycota are regarded as derived from algal ancestors and are separated from the Eumycota or "True Fungi". There are nine orders recognised under this class including Leptomitales, Saprolegniales, Pythiales and Peronosporales.

The Eumycota or "True" Fungi represent the largest group with over 4900 genera and 56000 species. They are eukaryotic organisms without plastids and their nutrition is absorptive. Their cell walls contain chitin and β-glucan. They are unicellular or filamentous multicellular and their spores are mostly non-flagellate or if flagella are present (as in the aquatic chytridiomycetes) they lack mastigosomes. The fungi reproduce asexually as well as sexually and the diploid phase is generally short lived. Four phyla are recognized under the Eumycota, viz. Ascomycota, Basidiomycota, Chytridiomycota and Zygomycota. It will be obvious that the presently accepted classification is at variance from the classification that would be familiar to most students of mycology from the earlier classical textbooks. The term Phycomycetes is now abandoned since on the basis of recent data the aquatic chytridiomycetes belong to Eumycota whereas the rest of the groups of aquatic fungi belong to Oomycota with affinities to photosynthetic algae (Chromista). Likewise the so-called "Fungi Imperfecti" or Deuteromycotina has not been accepted as a formal taxonomic category. They are not a monophyletic unit, but fungi which have either lost a sexual phase or represent anamorphs of other phyla (mainly Ascomycota and Basidiomycota). The terrestrial non-zoosporic fungi belonging to the Mucorales and Entomophthorales have also been classified under Eumycota as a separate phylum, Zygomycota. The slime moulds represent a class of fungi which have been separated under Protozoa, equal in rank with Chromista and Eumycota. Four phyla are recognized, viz. Acrasiomycota, Dictyosteliomycota, Myxomycota and Plasmodiophoromycota.

Taxonomic observations and interpretations on the identity of fungi have been strongly based on the morphological features. **H.H. Burdsall** (Mycologia 82:1–8, 1990)in his presidential address to the Mycological Society of America stated as follows: "Use of fungi in various manners brings the taxonomist to the fore because he is able to identify the fungus that is important to a process and can suggest related organisms that might be of use." However it is becoming increasingly evident that there is an overlap of morphological features among fungi which renders it difficult to get a clear resolution and proper identification in many instances based entirely on morphological basis. Species are taxonomic entities and are central to any discussion on biological diversity and in the absence of conceptual clarity in defining the limits of species, defining numbers of species identified lacks meaning. Also when field collections of fungi are made, the taxonomic description is based on the morphological features of the spore forms studied in a particular collection. Especially among the Hyphomycetes, the conidial (anamorph) stage and the telomorph or perfect state of the Ascomycete or Basidiomycete require to be bridged through careful cultural studies and in most cases when the two stages have been independently described, dual nomenclature for the same fungus prevails. Conventionally, the name assigned to the sexual stage (telomorph) is given to the fungus and the asexual or anamorphic state is regarded as its synonym. Among industrially important fungi for example the gibberellic acid producing fungus is an Ascomycete, *Gibberella fujikuroi* having the *Fusarium moniliforme* conidial state and the latter state is the one most extensively studied and used for initiating the fermentation studies. Likewise in the case of *Neurospora crassa*, the ascus stage is only produced in perithecia when opposite mating types are crossed, while the cultures used for microbiological assays or other industrial applications have only the asexual (conidial) state of *Monilia*.

The influence of environmental factors on the morphological features is often unaccounted in descriptions based on morphology alone. For example, Basidiomycetes such as *Ganoderma* are differentiated on the basis of the size of the

basidiocarps and also the ratio of the stipe length to size of the cap. Careful investigations under field conditions have shown that light intensity as well as the levels of carbon dioxide in the atmosphere have a significant influence on the dimensions of the fruit bodies and as such species designations based on collections from different locations may tend to be considered doubtful. When morphological data are only included, one should refer to it as 'morphological species concept'. The view point is the oldest and for practical and technical reasons many mycologists still base their species differentiation only on the basis of phenotypic characters.

Mycologists collect and study fungi from the natural environment and the emphasis of their study directly relates to their interest and specialisation. Mycological taxonomists observe the morphological characters of pathogenic or saprobic fungal specimens focussing on the spore morphology and the manner of spore development to identify novel features, necessitating description of their collection as a new taxon. Physiologists and biochemists would essentially relate their efforts towards establishing in vitro pure cultures for undertaking investigations on their metabolic pathways and metabolites with novel features and / or industrial potential. Fermentation technologists have mostly dealt with fungi capable of rapid growth and sporulation on simple nutrient media, which make them easily amenable for large scale cultivation and process scale-up. Species of *Aspergillus*, *Penicillium* and *Mucor* have been among the most investigated species finding favour with fermentation technologists. However current trends in the exploration of fungal biodiversity for valuable bioactive metabolites have started looking at pure cultures of a wide spectrum of taxonomically diverse fungi.

The Fungi Imperfecti or Deuteromycotina lacking the sexual stage and reproducing asexually by formation of abundant conidia have received greater attention because of their relative ease of cultivation and scale up besides ensuring genetic stability by virtue of the lack of a sexual phase which invariably results in recombination of characters and genetic instability in the progeny. The term "mitosporic fungi" has

been applied for these conidial Hyphomycetes (Dictionary of Fungi, 282–283, 1995), which are defined as "anamorphic (conidial) states of those fungi whose meiotic states (sexual state, telomorph) are not known and where known, these are related either to the "perfect" states in either ascomycetes or basidiomycetes."

Three classes of mitosporic fungi are recognised and these are as follows:

- Hyphomycetes: These are mycelial forms which bear asexual spores on separate conidiophores which may be aggregated in synnemata or form cushion-like sporodochia which are open and not enclosed inside a shallow or deep pycnidium.
- Aganomycetes or Mycelial Fungi (Mycelia Sterilia): These are fungi lacking conidia but may be forming chalmydospores or sclerotia by aggregation or thickening of the participating hyphae.
- Coelomycetes: These are fungi in which the asexual spores are borne inside well defined and enclosed structures called pycnidia or acervuli and when fully enclosed, has a well defined pore (ostiole) through which the spores are released.

Species definition is vital to any discussion on biological diversity and needs to be defined precisely on the basis of conceptual clarity on the differentiating traits that will define them individually. In fungi, the spore morphology and the manner in which the spores are produced have been the essential criteria for classification as well as generic and specific identification. Identification and description of new genera and species has been an intellectual activity of enthusiastic classical mycologists and it would be no exaggeration to attribute the progress in our knowledge of fungal diversity to these painstaking investigations and contributions. In dealing with the wide range of diverse fungi, taxonomic interpretations have, from time to time, come under controversies regarding the limits in definition of genera and

species and such nomenclatural changes proposed in the literature leave the non-specialists utterly confused.

Currently correct identification and taxonomy commands more than academic interest with the patenting of biotechnology processes getting a boost the world over along with the cultures of the organisms gaining importance in the synthesis of various biomolecules, correct nomenclature of the original strain(s) is obligatory. Besides, closely related strains which are classified as related due to morphological similarities may require better resolution for biotechnological applications. For example, *Aspergillus oryzae*, which is widely used for production of fungal alpha amylase used as a digestive aid in pharmacology ("Taka Diastase") and *Aspergillus flavus* producing the highly toxic aflatoxins are morphologically very close. While selecting the strain for the production of pharmaceutical amylase, it becomes necessary that they are established to be totally free from aflatoxin production even at extremely low levels. Mutation of strains for hyperproduction of desired metabolites using various physical and chemical mutagens has resulted in the selection of strains totally at variance in morphological features from the parental wild strain. For example, the high yielding penicillin producers of *Penicillium chrysogenum* often produce colonies with white sporulating structures and lack the yellowish diffusible pigment accompanying the bluish green spores of the parental strain.

It is obvious from the foregoing discussion that there is need for a better resolution of species definition and morphology alone may not be reliable enough to reach conclusions, especially in several critical situations. In recent years have seen several efforts in generating supplementary data on species designation based on biochemical parameters such as carbohydrate analysis, isozyme patterns, etc. Recent progress in molecular techniques have opened up several promising leads which if properly applied may offer meaningful parameters helpful in species resolution among fungi, which can be used for supplementing the data obtained through morphological and physiological studies.

Attempts to find suitable biochemical tools for differentiating taxonomic diversity among the fungi have resulted in different degrees of success. Indole secondary metabolites were considered for differentiating *Penicillium* species such as *P. camemberti* and *P. discolor* supplementary to morphological features (**Lund.** *Lett. Appl. Microbiol.* 20:228–31, 1995). Cellular fatty acids analysis of hundred fungi belonging to the different classes of Oomycetes, Zygomycetes, Ascomycetes, Basidiomycetes and Sterile mycelial forms revealed that many fungi produced the same fatty acids but produced different relative concentrations of each. Both culture temperature and age affected fatty acid composition, but when these factors were held constant, variance in fatty acid composition was not a problem and fungal fatty acid profiles could be differentiated statistically (**Stahl** & **Klug.** *Appl. Env. Microbiol.* 62:4136–46, 1996). Designing molecular probes to uniquely recognize fungi at nearly any taxonomic levels is projected to make significant contributions to a better comprehension of natural biodiversity with improvements in their construction for offering obvious advantages of rapidity, simplicity and amenability to automation (**Peterson.** *Chem. Abst.* 125:189651–d, 1996).

In mycological literature, instances of taxonomic confusion have arisen from time to time when an interesting metabolite of value is identified in different strains and the individual workers have assigned different names to the producing cultures. For example, the biocontrol agent produced for control of root disease fungi has been termed viridin or gliotoxin on the basis of the authors designating the fungal isolate as *Trichoderma viride* or *Gliocladium* sp. Subsequent work has shown that the compounds are metabolites of both *Trichoderma* and *Gliocladium*. Such examples would indicate the necessity to exercise the utmost caution in interpreting the utility of the secondary metabolites as basis for differentiating taxonomic identity of fungal cultures (**Mehrotra.** *Kavaka* 25:1–10, 1997).

In recent years, molecular biology techniques have provided newer insights into the evolutionary relationships among taxa

in the biological systems. It is but logical that this has stimulated studies towards their application to fungal systematics and these results have provided a useful tool to add a new dimension to view the phylogenetic relationships among fungal taxa. The earlier molecular studies in fungal systematics involved the measurement of DNA base composition, DNA:DNA or RNA:RNA relatedness through in vitro hybridisation and Restriction Fragment Length Polymorphism of mitochondrial DNA (mt DNA RFLP). DNA:DNA hybridisation studies have focused on the percentage of cross hybridisation between total DNA extract and the most interesting observation made has been that the percentage of DNA cross hybridisation between even closely related species is exceedingly low (less than 2%) whereas percentage cross hybridisation between individuals within a biological species is typically greater than 90% (**Bruns** et al. *Ann. Rev. Ecol. Syst*. 22:525–64, 1991).

In fungi, intraspecific genomic variations have also been studied by electrophoretic karyotyping and by the Random Amplified Polymorphic DNA (RAPD) method which takes advantage of the Polymerase Chain Reaction (PCR) technology. PCR has provided accessibility to many genes for study and has also provided an alternative to tedious and cumbersome cloning procedures. In future years, PCR technology can be expected to have significant and greater impact in fungal systematics studies. Application of RAPD using PCR technology for efficient elimination of duplicate strains in microbial screening for metabolites has been advocated by **Fujimori** & **Okada** (*J. Antibiotics* 47: 173–82, 1994). Taking the example of *Trichoderma* in which species are difficult to distinguish on the basis of morphological features alone, these authors showed that the electrophoretic band patterns of the PCR products gave results consistent with the morphological, physiological and ecological data on these strains. The electrophoretic band patterns of 74 strains of *Trichoderma* correlated well with their morphological and cultural properties, metabolite production profiles and ecological data. They concluded that RAPD is a simple,

efficient and reliable method for the selection of fungal strains employed in screening for bioactive metabolites.

Ribosomal RNA (rRNA) and ribosomal genes (rDNA) have been studied for their usefulness in fungal systematics. It is accepted that ribosomal genes are well characterised, ubiquitous and easily accessible via PCR technology. In ribosomal DNA sequencing, intergenic transcribed spacers (ITS) and intergenic regions (IGR) are more variable than the coding regions, and these variable rDNA regions could offer valuable guidelines for characterising and differentiating between two closely related species. It is possible that the ITS/IGR ratio could be suitable for designing species specific oligonucleotide probes for fungal identification. Based on DNA sequence analysis or internal transcribed spacers of ribosomal DNA, phylogenetic studies have been carried out with important plant pathogenic fungi such as *Phytophthora* (**Lee** & **Taylor.** *Mol. Biol. Evol.* 9:636–53, 1993) and *Puccinia* (**Zambino** & **Szabo.** *Mycologia* 85:401–14, 1993). **Lee** et al. (*Phytopathology*, 83:177–81, 1993) have designed synthetic oligonucleotide probes from ITS sequence data to distinguish between different *Phytophthora* species.

From the foregoing brief outline of the recent studies on the use of molecular techniques for a better characterisation of strains taxonomically, it is obvious that newer avenues are being explored vigorously to make fungal taxonomy more meaningful. Several biochemists and molecular biologists believe in and recommend molecular taxonomy as the only realistic and reliable basis for classification and identification of fungi. Will it be possible to dispense with morphology-based taxonomy and fully switch over to molecular taxonomy and achieve reliable classification and identification? Let us consider the possibilities.

A careful analysis will show that while molecular data could contribute useful attributes for a better understanding of the phylogeny and evolutionary relationships of fungi, taxonomy and classification based on morphology would still be the most readily applicable and practical means for classification and differentiation of fungal strains. **Hawksworth** (*Mycologist*

11:18–22, Feb. 1997) points out that it may not be realistic to expect molecular approaches to be the key for fungal classification. "As about 1800 fungi new to science are described every year and less than 100 species are sequenced each year, the gap widens rather than contracts. Technological developments including automated molecular and other biochemical procedures are unlikely to be of practical value for many years, except in certain fungi of medical and agricultural significance."

It can be safely assumed that in the immediate future at least, industrial biotechnology and fermentation studies for fungal-based biomolecules will have to primarily rely upon morphology-based taxonomy for discovering novel strains, while exploring natural biodiversity. Further advances in molecular techniques may see the advent of an era in which molecular biology would play increasingly significant role, not only in aiding fungal taxonomy, but also in the augmented expression of heterologous genes and eukaryotic proteins in fungal systems through recombinant DNA technology.

CHAPTER 2

Selective Isolation of Fungi

Biotechnological Applications

The widespread distribution of fungi colonising various natural organic substrates and the limitations in our knowledge on the various ecological niches which harbour specialised groups, make it necessary to undertake detailed investigations leading to pure culture isolation, especially of the rarer and biotechnologically less-investigated taxa. Based on our current knowledge about the ecology and distribution of different fungi and by selecting specific habitats by providing optimal conditions for growth, it would be possible to screen for select taxonomic groups and build a germplasm collection for biotechnological explorations. From the viewpoint of an industrial biotechnologist while screening fungal strains from the natural environment, three factors have to be borne in mind:

1. Screening for rare or little investigated taxa based on the optimism that they represent novel gene pools for biomolecules with novel features.
2. Screening should be done for taxonomically related genera and species to those with established biotechnology potential.
3. Adopting innovative and selective procedures for getting access to the desired taxa should be based on a sound biochemical knowledge related to the metabolic pathways which are involved in biomolecule production.

All these three aspects are overlapping and integral to screening and are not to be regarded as separate and distinctive. In step with the modern trend of thinking, the gene pools carried by every microbe is invaluable for biotechnological inventions and deserve to be explored and conserved for detailed investigations. Presently fungal taxa belonging to every taxonomic group is included in screening programs and even plant pathogenic fungi, earlier considered only from the point of their control measures, are included for biometabolite screening. Examples of valuable metabolites obtained from plant pathogenic fungi include lysergic acid and ergot alkaloids from species of *Claviceps* such as *C. purpurea* and *C. paspali*, riboflavin from *Eremothecium ashbyii* and *Ashbya gossypii* which are pathogenic to cotton and ustilagic acid, a synthetic musk precursor produced by the smut fungus *Ustilago maydis*. The source of gibberellic acid widely used as a plant growth hormone is also a pathogen of rice plant, *Gibberella fujikuroi* (Anamorph: *Fusarium moniliforme*).

In the natural environment, fungi colonise different organic substrata and co-exist along with other microflora and it is necessary to realise that they vary in their relative abundance, rate of growth and nutritional fastidiousness. In order to identify and isolate the pure culture of the relatively less abundant and slower growing species, special techniques are required to be employed wherein competition from the more abundant and faster growing species can be minimised, if not fully eliminated. In devising the techniques for accomplishing this, all the three aspects related to isolation, referred to earlier would be applicable.

In this chapter, some of the useful techniques employed for the isolation of different groups of fungi, including selective techniques, media formulations and incubation procedures are discussed. The techniques discussed can provide the industrial biotechnologist an insight into the diverse alternatives and approaches available for making a meaningful inventory of fungal biodiversity from natural substrates.

Collection of Samples

Field collections of fleshy fungi like mushrooms and polypores can be made during the monsoon months and these require to be processed immediately for establishing cultures. Herbaria and/or preserved specimens and field notes on their descriptions in the fresh state will be useful in their identification. It is advisable not to collect material for culturing immediately after heavy rains since such material offer serious bacterial contamination problems. Relatively dry surfaces on the fruit bodies are more suited for the establishment of pure cultures, which may be initiated from the spores or more readily by aseptically transferring portions of the fruit body after mild surface sterilisation on suitable agar media. Field collections of fleshy fungi are preferably stored in paper bags. Plastic bags should not be used for their storage.

Soil samples, dung and plant litter are collected and allowed to air dry before their storage in paper bags recording details of the date of collection, location and any other interesting field observations. It is advisable to process them as soon as possible since many fungal spores lose viability under storage conditions and may not be easily recoverable. Some Ascomycetes and Deuteromycetes are capable of withstanding storage up to six months of freezing at –20 °C. It would be worthwhile to freeze small quantities of the collections for further processing to isolate these groups from them.

Water samples collected from lakes or streams also require to be processed immediately for recovering aquatic fungi. Decomposing leaves collected from the bottom of shallow fresh water habitats are rich sources of aquatic hyphomycetes and are preferably examined immediately after collection and culture work started on them. From the marine ecosystem, apart from mud samples and sea water, fungi specific to such habitats can be isolated also from decomposing drift wood samples, marine algae, flowering plants and also sponges and crustacean debris.

Culture Media for Isolation

A variety of mycological media have been studied for isolation of fungi from natural substrates. Most of the fungi grow best in media rich in carbohydrates and under slightly acidic pH. However the media pH can be adjusted to around 6.0–6.5 and the bacterial contaminants in isolation plates can be successfully controlled by incorporating a broad spectrum antibacterial antibiotic such as tetracycline at 10–25 µg/ml, after sterilisation of the media and prior to the plating of the samples. Many workers prefer a weak nutrient medium such as Hay infusion agar or potato carrot agar for isolation, since the radial spread of the fungal colony will be restricted and the fungi will also sporulate more rapidly due to nutrient limitations. Other media employed are potato dextrose agar, malt extract agar, Emerson's agar, corn meal agar or Leonian's medium. Depending on the modifications implemented by way of specific chemicals and / or antibiotics as well as the pH and temperature of incubation, select groups of fungi can be recovered and isolated in pure culture.

Aquatic Zoosporic Fungi

The unicellular Chytridiomycetes as well as the filamentous Oomycetes are present in aquatic habitats and are characterised by the formation of free swimming zoospores. Sexual reproduction is through male and female gametes formed inside gametangia in the filamentous Oomycetes belonging to orders like Saprolegniales and Leptomitales. Chytrids from aquatic habitats can be allowed to develop by dropping autoclaved pollen grains of pine by what is referred to as baiting technique. On the baits, such as pine pollen or cellulosic baits such as tissue paper as well as hair or snake skin, aquatic fungi will selectively develop which are capable of utilising the nutrients provided by these substrates. By observing and transferring a pollen grain with a ripe sporangium of a chytrid to sterile water and adding new pollen

grains, it will be possible to establish a culture almost in pure state of the specific chytrid. Rolling the pollen grains on hard (3%) agar containing 0.15% maltose and 0.004% peptone, the chytrid could be established in pure culture on agar media. **Stanier** (*J. Bact*. 43:499, 1942) described a technique for isolation and culture of the cellulose decomposing chytrid, *Rhizophlyctis rosea*. It consists of pouring a mineral agar plate and seeding it with cellulose in the form of filter paper or lens paper and 0.5% glucose and inoculating it with compost. The mineral medium contained 0.1% each of potassium phosphate (dibasic) and ammonium sulphate, 0.02% magnesium sulphate and 0.01% calcium chloride and trace quantity of ferric chloride. The filter paper showed a pink growth of the chytrid after four days and pure stock cultures were maintained on mineral glucose agar slants or in liquid mineral base with bits of filter paper partly immersed. Transfers were accomplished by simply flooding the agar slants less than a week old with water and streaking resultant zoospore suspensions on agar. If older cultures on cellulose media were used, pieces of agar or cellulose were removed and placed in sterile water blanks. **Ajello** (*Amer. J. Bot*. 35:1–12, 1948) described the isolation of the chytrid, *Polychytrium aggregatum* from soil on chitinous baits. In pure cultures, the chytrid grew well on a medium containing 1% dextrose, 0.1% yeast extract, 0.1% Bacto Peptone and 2% agar. **Emerson** and **Cantino** (*Amer. J. Bot*. 35:151–71, 1948) found that fruits of rosaceous plants such as apple were capable of being used successfully as baits facilitating colonisation by species of *Blastocladiella* such as *B. pringsheimii* and *B. ramosa*. Pure cultures could be established on a synthetic salts medium containing 0.3% glucose and 0.1% Difco yeast extract.

A very widely used bait for isolating filamentous Oomycetes from aquatic habitats has been boiled hemp (*Cannabis sativa*) seeds. Boiled hemp seeds are gently squeezed to release the cotyledons and added to water samples or to soil samples flooded with activated charcoal treated water. Zoospores of aquatic fungi readily colonise the hemp seed baits and establish the mycelial growth along with the reproductive structures. In mycological literature many species have been

described on the basis of hemp seed cultures. Seeds of mustard (*Brassica nigra*) similarly boiled and used also have been found to enrich cultures of saprolegniaceous fungi like *Achlya* species. Pure bacteria-free axenic cultures can be established by streaking zoospores on media such as 2% cornmeal agar or bean pod (5% w/v) agar media. Bacterial contamination can be suppressed by addition of 5–10 µg/ml of streptomycin or tetracycline. Keratinophilic and chitinophilic species of zoosporic filamentous fungi can be obtained by baiting with autoclaved hair or snake skin, termite wings, etc. For example **Kane** (*Mycolgia* 58:905–11, 1966) isolated the keratinophilic *Leptolegniella* from soil by the following procedure: Five grams of soil are flooded with charcoal treated water in a petri dish and baited with autoclaved human hair. The saprolegniaceous keratinophilic fungus was observed after one week colonising the hair and forming both zoosporangia and male and female gametangia. Likewise species of *Aphanomyces* can be recovered from soil by baiting with snake skin boiled in water. These are just a few specific examples of techniques that are useful for the isolation and pure culture studies of zoosporic fungi. These groups of fungi have not received sufficient attention so far from biotechnologists but in the current philosophy of screening fungal biodiversity for biotechnological innovations, these fungi may also be expected in future to contribute their share to the discovery of novel metabolites and useful molecules.

A group of marine zoosporic fungi which is biotechnologically important has been classified in the genus *Thraustochytrium* which is isolated from sea water. The thraustochytrids synthesise an unusually high proportion of Polyunsaturated Fatty Acids (PUFA) and are potentially important for commercial applications.

The truly anaerobic rumen inhabiting chytrids represent a specialised group of zoosporic fungi that require special techniques for culturing. **Orpin** (*J. Gen. Microbiol*. 91:249–62, 1975) isolated *Neocallimastix frontalis* from sheep rumen by overlaying sloppy agar medium which contained antibacterial antibiotics with a sample of rumen digest. After incubation, the top layer of the culture was removed by aspiration and the

lower layer which contained the zoospores was overlaid on to fresh sloppy agar medium. The culture was then shaken gently to distribute the zoospores throughout the upper layer of the medium and was again incubated. The fungal biomass which was formed in the top layer of these cultures was transferred to fresh medium and the whole procedure was repeated until pure cultures were obtained. Other workers have followed the procedure of straining rumen fluid through muslin cloth mixing the filtrate with molten agar medium containing antibiotics and preparing Hungate Roll tubes as practised for cultivation of anaerobic bacteria. **Lowe** et al. (*J. Gen. Microbiol*. 131:2225–28, 1985) studied growth of anaerobic fungi on defined and semi-defined media lacking rumen fluid by following strictly the techniques employed for the culture of anaerobic bacteria. For preparation, storage and inoculation, an agar plate method was developed for successful cultivation of the anaerobic Chytridiomycetes.

Aquatic Hyphomycetes

Aquatic Hyphomycetes are widely distributed in foam samples, particularly around barriers such as fallen logs in rapidly flowing fresh water streams and also in decaying leaves beneath the water in clear ponds, shallow water pools etc. These spores are characterised by radiating arms or appendages which help them for buoyancy and also for anchoring to substrates in the flowing environment. This group is also characterised by the peculiar ability to sporulate under submerged conditions. The decaying leaves collected in stream are brought to the laboratory, washed and covered with a shallow layer of water in a petri dish. Incubation at 20–24 °C is optimal for the production of conidia by the aquatic hyphomycetes present in the specimen. Culture is established on simple agar media such as 2% malt extract agar. For observing sporulation, a strip or disk is cut out from the zone of active mycelial growth in the colony and submerged under sterile distilled water in a petridish. From the cut surface of the mycelial growth large numbers of conidiophores emerge

and develop the conidia at the water miniscus. The extent of conidial development varies widely with the individual species and strains. Some of the cultures have been successfully grown and conserved in vitro for long terms but others have posed difficulties in culturing as well as conservation with viability and genetic stability. While mycological descriptions based on spore characteristics from field collections are widespread in literature, this group has not received much attention with respect to its biotechnology potential. Extensive cultural studies and exploratory research for studying these fungi for their bioactive metabolites and enzymes would be worth undertaking.

Isolation of Fungi from Soil

Soil as a reservoir for a wide variety of pathogenic and saprophytic fungi has been recognised for a long time. Early studies have focussed on the survival of plant pathogenic fungi, particularly those infecting roots, under the adverse environmental conditions and when the crop is not present in the fields. In more recent years, attention has also been turned to the study of the saprophytic mycoflora for their antagonistic potential to destructive pathogens and also for their potential as sources of useful bioactive metabolites and industrial enzymes.

Studies on soil fungi have revealed that they comprise genera and species widely varying in their taxonomic affinities as well as in their physiological and nutritional requirements. They co-exist with other microflora colonising organic substrates and producing spores to propagate and also to survive the adverse climatic changes. Their extent of distribution and also their competitive saprophytic ability are widely varied. The frequency with which they can be isolated in pure culture is governed by the relative abundance of their propagules, rate of growth and nutritional fastidiousness. Thus rapidly growing, abundantly sporulating and nutritionally nonexacting fungi like *Aspergillus* and

Penicillium would rapidly appear on isolation plates and often may deprive the slower growing and less abundant species an opportunity to grow. Rapidly spreading cultures such as *Trichoderma* or *Neurospora* would so rapidly over-run the agar surface that it would be impossible to select the other colonies and purify them from such isolation plates.

It is evident from the above description that in order to explore and understand the extent of biodiversity of fungi in a soil sample, innovative techniques require to be applied. Different media formulations, enrichment techniques or other approaches should be tried in order to permit the slower growing and rarer species to grow overcoming the competition from the faster growing and abundant species.

Baiting with specific substrates has been successfully used for isolating fungi which would not otherwise be readily isolated in plating out experiments. Soil particles are moistened with water or a dilute mineral salts solution and baited with autoclaved human hair or wool and incubated at optimum temperature, usually 28–30 °C for tropical species. Hair contains the scleroprotein, keratin, which is not readily utilised by most fungi, whereas the dermatophytes such as *Trichophyton* and *Microsporum* are able to attack and degrade the keratin and find the hair as a favourable substrate for growth. When a soil sample has populations of these fungi, the hair would be colonised and appear as a whitish growth mainly consisting of sporulating hyphae growing on the hair surface. These spores can be transferred to media like Sabouraud's agar supplemented with antibiotics to establish the dermatophytes in pure culture. Likewise when baited with chitin-rich substrates like termite wings or snake skin, chitinophilic fungi like *Chrysosporium* will get enriched on the substrate baits and from these pure cultures, the respective fungi can be readily isolated on agar media. It is sometimes better to give a mild solvent extraction for the hair, wings etc. before autoclaving in water. This is recommended to remove the associated lipid materials, since competition from fungi like aspergilli which can grow on the lipid material would impede the isolation of specific keratinophilic and chitinophilic strains. After solvent extraction, the baits are autoclaved in

water, blotted on filter paper to remove excess water and laid on the moistened soil surface. Free moisture films associated with the baits will tend to encourage bacterial growth and interfere with the colonisation of the desired fungi.

Plating out soil samples on agar media is a routine procedure followed to isolate fungi from soil. A 1:10 dilution in water is serially diluted and plated out in nutrient media adjusted to pH 4.0 after autoclaving or preferably by incorporating 10–25 µg/ml of a broad spectrum antibiotic like tetracycline to prevent bacterial growth. When serial dilutions of the supernatents of soil suspensions are plated the majority of fungal colonies that develop are derived from spores of heavily sporulating and abundantly distributed genera such as *Aspergillus*, *Penicillium* or *Mucor*. Feebly sporulating as well as mycelial forms are not easily isolated during the routine plating procedure. **Warcup** (*Nature* 166:117–18, 1950) proposed the soil plate method of fungal isolation from soil. A soil plate is prepared by transferring a small amount of the soil to be examined into a sterilised petri dish and 8–10 ml of cooled medium is poured over it. The soil particles are uniformly distributed by gently rotating the agar before solidification. Usually 5–15 mg soil per plate gives a convenient number of colonies on each plate. Czapek-Dox agar supplemented with 0.05 to 0.5% yeast extract was found to be a satisfactory medium for isolation studies. The soil plate method yielded a wider range of species compared to plating out of the suspensions. Apparently shaking the soil with water does not allow all the fungi present to pass into suspension and hence isolation plates which incorporate the soil particles enable a wider range of species to be isolated compared to the dilution plates. **Warcup** (*Nature* 175:953–54, 1955) described a procedure for isolation of fungi from hyphae present in the soil and it is noteworthy that many of these forms were not obtained in soil dilution plates. The procedure was outlined as follows: 1–1.5 gram soil crumb is placed in a beaker partly filled with water and allowed to become saturated. After 4–5 minutes, the crumb is broken apart by filling the beaker with a rapid jet of tap water. The heavier soil particles are allowed to settle for 1–1.5 minutes and then most of the supernatent is

poured off. Further water is added, the heavier particles allowed to sediment and the suspension again removed. The procedure is continued repeatedly until the liquid remains clear after standing for 1–1.5 minutes. The soil particles in the residue are then distributed in a small quantity of water among three sterile petri plates and searched for the presence of fungal hyphae by examining under a binocular dissecting microscope. Using a fine forceps, individual hyphae or portions of hyphal masses are carefully removed and transferred to a drop of sterile water in a petri dish. Removal of as much of the organic particles attached to the hyphae as possible is carefully carried out followed by plating out with melted cooled agar. From the well dispersed fungal hyphae colonies develop. The medium recommended is Czapek Dox–yeast extract agar (pH 5.6–5.8) diluted to one sixth of its normal strength. The diversity of forms successfully isolated by this method include several mycelial Basidiomycetes as well as other non-sporulating species and as already mentioned these forms were not isolated by the normal dilution plate methods. The direct hyphal method successfully isolates a large group of soil-inhabiting mycelial fungi hitherto totally neglected in the studies on soil fungi.

In a further modification of the soil plate isolation technique, **Warcup** and **Baker** (*Nature* 197:1317–18, 1963) showed the presence of dormant spores in soil, especially ascospores of ascomycetous fungi which are activated by heat and devised a technique facilitating their isolation. 2.5 grams of soil was immersed in 60% alcohol for 6–8 minutes and then the soil and alcohol added to a water blank to give a dilution of 1:100 (and an alcohol concentration of less than 1%). 1 ml portions of the suspension were taken after thorough mixing and heated at 60 °C for 30 minutes in a water bath. Dilution plates were prepared with Dox-yeast extract agar containing 30 µg/ml of streptomycin and 2 µg/ml of aureomycin to suppress bacterial growth. The alcohol "pasteurisation" and heat treatment while successfully eliminating the common and rapidly growing forms, facilitated the isolation of several ascomycetous fungi. Alcohol treatment of soil samples has been successfully employed by many workers for the isolation

of rare fungi like the mucoralean genus *Mycotypha* (*M. africana*) from African soil in which the zygospores must have survived the alcohol treatment (**Novak** and **Backus**. *Mycologia* 55:790–98, 1963), or *Aspergillus longivesica* (**Huang** and **Raper**. *Mycologia* 63:50–57, 1971).

Use of Selective Media Formulations

Several innovative approaches have been practised in an attempt to provide optimal growth conditions for the slower growing species and many of these are based on knowledge related to the physiology and biochemistry of individual fungal groups. Several chemicals and antibiotic combinations have been successfully employed to ensure recovery of select groups on the isolation plates from natural substrates screened. The subject has been reviewed and may be consulted for further details (**Vaartaja**. *Phytopathology* 50, 870–73, 1960, **Tsao**. *Ann. Rev. Phytopathology* 8, 157–86, 1970). Formulation of selective media is generally based on the use of chemicals and/or antibiotics at concentrations which inhibit some fungi but are not inhibitory to others. This provides unlimited opportunities for manipulation of the culture medium employed for isolation purposes, while incorporation of one or more antibacterial antibiotics prevents competition from bacterial colonies. For example, polyene antifungal antibiotics like nystatin inhibit hyphomycetous fungi by binding to their sterols, while phycomycetous fungi lacking sterols in their cell walls are not inhibited by the polyenes. **Hendrix** and **Kuhlman**. (*Phytopathology* 55:1183–87, 1965) used an isolation medium supplemented with 100 units per ml of Nystatin, 100 ppm of pentachloronitrobenzene (PCNB), 50 ppm of Streptomycin and 60 ppm of rose bengal for preferential isolation of *Phytophthora* from soil samples. Acti-Dion (cycloheximide) exerts higher antifungal activity on common moulds like aspergilli and penicillia, which are inhibited at lower concentrations, whereas many plant pathogenic and human pathogenic species are not inhibited even at higher levels of the antibiotic. This differential

sensitivity has been taken advantage of and plant pathogenic species of *Ceratocystis* have been successfully recovered from infected tissues overcoming saprophytic fungal contaminants on media containing 200 ppm cycloheximide and 10 ppm streptomycin (**Schneider**. *Plant Disease Reporter* 40: 516–21, 1956). Selective media for the isolation of basidiomycetous fungi from soil as well as woody materials have used chemicals exerting greater inhibitory effects on common saprophytic fungal flora. For example, o-nitro phenol at 60 ppm inhibited fast growing *Trichoderma* and facilitated isolation of *Fomes annosus* and other Basidiomycetes from wood pulp (**Russell**. *Nature* 177:1038–39, 1956). *Phanerochaete chrysosporium* is a white rot Basidiomycete which as already mentioned in Chapter 1 has also shown the ability to mineralise several xenobiotic compounds including toxic chlorinated phenols. The ability of this fungus to degrade these normally recalcitrant compounds makes it an attractive candidate for bioremediation of soils contaminated with hazardous organic materials. **Dietrich** and **Lamar** (*Appl. and Environ. Microbiol.* 56:3088–92, 1990) devised a selective medium for isolating this fungus from soil. The selective medium contained 15 ppm benomyl (1-butylcarbonyl-2-benzimidazole carbonic acid methyl ester) (50% wettable powder dissolved in acetone) and 550 ppm of streptomycin sulphate in 2% malt agar and incubated at 39 °C. Under these conditions *P. chrysosporium* was recovered from soil overcoming competition from other microorganisms. A review on selective agar media for the isolation of Basidiomycetes from wood has been published by **Halle and Savory** (*Int. Biodeterioration Bull.* 12:112–15, 1976).

Suppression of fast growing fungi to enable isolation of the relatively slower growing species has been achieved by incorporating growth retardants in the medium. Rose Bengal has been used by several investigators. **Curl** (*Can. J. Microbiol.* 14:182–83, 1968) reported that 500 µg/ml of plant growth retardant Phosfon (2, 4-dichloro benzyl-tributyl phosphonium chloride) significantly increased the numbers of colonies per plate and suppressed fast growing fungi. Likewise Dichloran (2,6-dichloro-4-nitroaniline) added at 5–25 mg per

litre suppressed the growth of aspergilli and penicillia (**King** et al. *Appl. Env. Microbiol*. 37:959–64, 1979). Other compounds which have been added to retard colony spread include oxgall (0.5–1.5%) and pentachloro nitrobenzene (PCNB). The latter compound is carcinogenic and hence is not recommended for routine use in the laboratories.

Screening for fungal strains utilising complex substrates like carbohydrates and proteins and exploring them as sources of industrially useful enzymes are major initiatives in biotechnological research. Cellulose degradation by cellulolytic enzymes to produce glucose which can be fermented to ethanol is a dream widely projected for ensuring unlimited supply of a clean burning liquid fuel from renewable resources available in plant biomass. **Eggins** and **Pugh** (*Nature* 193:94–95, 1962) formulated a medium consisting of inorganic salts, asparagine (0.5 mg/ml) ball milled cellulose (10 g/l), and agar (2%) for isolation and enumeration of fungi able to decompose cellulose. The cultures were assessed for linear growth rate, mycelial density of colonies and clearing of the medium within the colony (destruction of cellulose). In a subsequent study, **Park** (*Trans. Brit. Mycol*. Soc. 60:148–51, 1973) reported that omission of asparagine gave significantly higher counts of cellulolytic fungi from garden soil as well as soil mixed with plant litter. **Okada** et al. (*Trans. Mycol. Soc. Japan* 34:171–85, 1993) isolated a new cellulolytic alkalophilic hyphomycete from sludge, and named it as *Acremonium* alcalophilum. The isolation medium consisted of mineral salts, 1.5% cellulose powder, 0.1% peptone and 0.05% yeast extract and 1.5% agar. To 900 ml of the above medium, 100 ml of 6% sodium carbonate was added separately sterilised, to get a pH of 9.2. On this medium, the alkalophilic fungus was isolated and successfully cultivated. In our studies at the National Chemical Laboratory, Pune, in the screening for cellulase-free, alkali stable xylanase with potential application in paper and pulp biotechnology, an alkalotolerant *Cephalosporium* was isolated on a wheat bran medium adjusted to pH 10.0 with sterile sodium carbonate and supplemented with 50 µg/ml of tetracycline. When cultured on xylan agar plates at high alkaline pH (9.5–10.0) the growing colonies showed marked

xylan clearance beyond the growing edge of the colony indicating xylanase secretion. A process patent for cellulase-free xylanase production by this fungal strain has been granted (**Rele**, **Bansod** and **Srinivasan**. U.S.Patent No.5534429, 1996).

Microbial production of Gamma-Linolenic Acid and other Polyunsaturated Fatty Acids (PUFA) has received considerable attention in recent years and species of mucoralean fungi, especially *Mortierella* have been studied for their production. **Botha** et al. (*System. Appl. Microbiol*. 18:448–54, 1995) formulated an isolation medium containing mineral salts and trace elements, 0.05% yeast extract and 20 g/l of sodium acetate. Acetate-tolerant mucoralean fungi capable of growing well on a medium containing 20 g/l of acetate mainly belonged to the genera *Actinomucor*, *Mucor* and *Thamnostylum*. It was found that these isolates compared favourably with strains representing species known for substantial accumulation of gamma-linolenic acid.

Isolation from Litter and Plant Residues

Plant litter and decaying plant residues including rotting wood harbour a range of fungal diversity which warrants much greater attention to be paid than is presently done. Moist chamber incubation and periodic examination under the microscope reveals a variety of fungi sporulating on the detritus. Pure cultures of these fungi can be established by directly transferring the spores with a fine needle to agar media. Weak nutrient media such as Hay infusion agar, bean pod agar or potato carrot agar are preferable to conventional sugar-rich mycological media for isolation and culture of litter fungi. Plant litter, immediately after collection from the field should be examined with a hand lens to observe the presence of fruiting structures like pycnidia, cleistothecia or perithecia. Such fruit bodies if sectioned in a drop of sterile water would release large numbers of the internally borne spores, which may be studied for their morphology and transferred to agar

media containing 10 µg/ml of tetracycline (added just prior to pouring after cooling to around 55 °C). Spore germination and colony development can be followed microscopically and pure cultures transferred to agar slants. Placing an autoclaved grass leaf on the weak medium slants and inoculating the young colony on it has often resulted in characteristic fruit body development on the grass leaf earlier than on the rest of the medium.

Bills and **Polishook** (*Mycologia* 86:187–98, 1994) in their study on the abundance and diversity of microfungi in leaf litter of a lowland rain forest in Costa Rica developed a particle filtration technique which yielded greater fungal diversity from litter. The procedure for particle filtration was described as follows: Air dried litter (5 grams) was placed in a sterilised Waring blendor and pulverised for one minute. The pulverised sample was then washed with a stream of distilled water for 10 minutes through a 2 mm brass screen and through two sterilised filters of 210 µm and 105 µm. The particles trapped on these filters were individually collected, washed, centrifuged and small portions spread on the surface of selective agar media. The plates were incubated under 12 hours fluorescent light photoperiod to stimulate sporulation. The particle filtration procedure brought into culture a wide range of taxa, many of which originated from the interior of fine litter particles freed from surface borne propagules, through repeated washing. The authors concluded that the particle filtration was an efficient method for rapid recovery of a large number of fungal species from plant litter of tropical forests.

One group of fungi which are widely distributed in decomposing plant litter but seldom encountered in plating out experiments is the saprophytic Entomophthorales, particularly the genera *Conidiobolus* and *Basidiobolus*. The large conidia are forcibly discharged into the air from unbranched phototropic conidiophores and readily germinate to form colonies. Young colonies produce numerous conidiphores and from these conidia are discharged beyond the growing edge and develop several daughter colonies. **Drechsler** (*Science* 115:575–76, 1952) described a technique

for isolation of these fungi, in which fine particles of detritus are mixed with soft agar on the inner surface of a petri dish lid and canopied on an agar medium like maizemeal agar. From the detritus entomopthoraceous fungi put forth aerial conidiophores and discharge the conidia on the agar and bacteria-free cultures are readily isolated. **Srinivasan** and **Thirumalachar** in a series of papers described *Conidiobolus* species including new species isolated from the Indian subcontinent and gave taxonomic keys for the species differentiation in *Conidiobolus* (*Mycologia* 59:695–713, 1967) and *Basidiobolus* (*Mycopathologia Mycologia Applicata* 33:56–64, 1967). *Conidiobolus* strains producing high activity alkaline protease compatible with commercial detergents and with potential applications in the leather industry have been identified during our studies carried out at the National Chemical Laboratory, Pune (**Phadatare** et al. *Enzyme Microbiol. Technol*. 15:72–76, 1993).

Another group of fungi widely distributed in leaf mould and forest litter is the predacious fungi capturing and destroying microscopic animals like amoebae and nematodes. These include Phycomycetes classified under the order Zoopagales which attack amoeba and hyphomycetous fungi which kill nematodes and belonging to the genera such as *Arthrobotrys*, *Dactylella* etc. Some of these hyphomycetous species have been grown in pure cultures and evaluated for their potential in controlling nematode populations in soil. In the present context of intensive search for newer antifungal agents based on interference with chitin metabolism, some of these predacious fungi may hold promise and biotechnological significance. Drechsler who worked extensively on predacious fungi isolated and identified them by seeding *Pythium* cultures growing on a weak (1–2%) maizemeal agar plates with finely powdered and sieved leaf mould and incubating them for several days. The nematode populations thriving on the agar plates eventually get infected and the fungi sporulates on the dead nematode worms. By carefully transferring the spores to fresh agar media, pure cultures can be established.

Isolation of Dung Inhabiting Fungi

It is well known that animal dung, particularly of herbivores supports a rich variety of mycoflora ranging in diversity from Zygomycetes to Ascomycetes, Basidiomycetes and Deuteromycetes. The succession of fungi on dung incubated in moist chambers for periods extending up to several weeks and isolating the cultures from the sporulating structures as they appear is rewarding from an ecological as well as biotechnological point of view. Ascospores of many coprophilous Ascomycetes are heat resistant and may require heating in the form of steam treatment prior to germination. Dung can be plated out following all the protocols and media formulations outlined under isolation of soil fungi and such studies would lead to recovery of a spectacular range of novel genera and species. **Webster** (*Ecol. of Fungi* edited by **D.J. Bhat** and **S. Raghukumar**, Goa University Press 1–11, 2000) pointed out the importance of relative humidity levels on the succession of coprophilous fungi on dung samples incubated in moist chambers. Rabbit dung pellets when incubated in moist chambers with either water or different saturated salt solutions to give a relative humidity ranging from 100% to 81%, the response of different fungi to grow was studied (**Kuthubutheen** and **Webster**. *Trans. Brit. Mycol. Soc.* 86:63–76, 1986). The fungal flora which was encountered under low relative humidities varied significantly from those which developed under high moisture content during an experiment carried out for a total period of sixty days.

Seed-borne Fungi

Seeds harbour a wide range of saprophytic and plant pathogenic fungi. Seed pathologists have standardised the protocols for incubating seed samples on moist blotters in plastic petri dishes and incubation under Near Ultraviolet Lamp for 8 to 12 hour photoperiods. While their interest is primarily to evaluate the percentage of seeds in a seed lot

carrying economically important phytopathogens (e.g. *Pyricularia oryzae* in rice seeds), the variety of saprophytic fungi developing on these incubated seeds hold great potential for bioactive metabolites and industrially useful enzymes. Seeds vary widely in composition and sometimes contain very unique compounds such as lectins, and these may exert selective pressures on the composition of the mycoflora that the seeds harbour. The aspect of exploring seed-borne fungi for biotechnological purposes such as discovering novel enzymes or for biotransformation of chemical molecules has so far received scant attention and perhaps intensification of research efforts to isolate and conserve fungal mycoflora of different seeds and exploring their biotechnology potential would be worthwhile.

Osmophilic Fungi

Osmophilic or xerophilic fungi are those which grow and survive under low water activity situations. Several of them, capable of growing on substrates containing high concentrations of sugar are responsible for spoilage of food stuffs. Isolation in pure culture of xerophiles involves the use of media with reduced *a*

reactions in the medium. Isolation is carried out by directly transferring bits of the material such as spoiled foods directly to the surface of agar and incubating to observe colony development from growth initiated out of the inoculated pieces. Isolation plates are preferably sealed with parafilm to minimise media dehydration. Species of *Aspergillus* such as *A. glaucus, A. tonophilus* can be isolated from natural substrates especially seeds in storage, where they sometimes cause considerable seed deterioration by virtue of their ability to grow under xerophytic conditions. These aspergilli grow poorly in normal mycological media but grow well on media containing 40–70% sugars like glucose or sucrose.

Thermophilic Fungi

Fungi growing optimally at temperatures above 40 °C and up to 45–55 °C are grouped under thermophiles. True thermophiles have their optimum temperature above 45 °C while a range of thermotolerant species grow well at 37–40 °C. They are abundant in self-heating organic matter such as wood chip piles, stored bagasse and compost pits. Successful isolation of thermophilic fungi on various nutrient media can also be made from soil, dung and tobacco products like snuff, bird's nest, etc. The ability to grow well at 37 °C which is the human body temperature make thermophilic fungi potentially capable of causing harmful reactions to humans. Industrial microbiologists should be particularly careful while handling the commonly encountered thermotolerant *Aspergillus fumigatus* with bluish green conidiophores and abundant conidial chains. *A. fumigatus* can cause severe allergies as well as lung infections in persons exposed to heavy spore populations. Several thermophilic fungi are important from a biotechnological point of view. These include *Chaetomium cellulolyticum* which can grow on pretreated cellulose-rich agroresidues such as wheat straw to produce a protein-rich mycelial biomass product for use in animal nutrition. Species of *Rhizomucor* like *R. miehei* and *R. pusillus* grow at high temperatures and secrete an acid stable protease with milk

clotting activity and has been shown to be useful in cheese manufacture. A recent review by **Maheshwari** et al. (*Microbiol. Mol. Biol. Rev*. 64:461–488, 2000) discuss in detail the various aspects related to the physiology and enzymes of thermophilic fungi.

From the foregoing extensive but not exhaustive account, an insight is provided into the variety of techniques available which can be applied meaningfully for a better understanding of naturally occurring fungal biodiversity. With the realisation that we have only a very small percentage of species available in pure culture and that the tropical ecosystem of our country would be a rich gold mine of fungal diversity, it is now up to the Indian mycologists and biotechnologists to undertake planned and serious exploration of indigenous mycoflora with the prime objective of building up a viable germplasm collection of authentically identified indigenous fungi. The opportunities and challenges that such a venture would offer to make innovative biotechnological studies, would certainly make such an exercise worthwhile.

CHAPTER 3

Culture Collections

Germplasm Resource Pools for Biotechnology Research and Development

The filamentous fungi growing in pure cultures and properly conserved represent a germplasm resource pool offering unlimited opportunities for discovering novel bioactive molecules and industrially useful enzymes. Culture collections, maintaining several thousand strains have been in existence for nearly a century, the oldest and one of the largest maintaining fungal cultures being the Centraalbureau voor Schimmelcultures, established in the Netherlands in 1904. Other major collections abroad which are repositories of diverse fungal cultures including "Type" strains are the American Type Culture Collection (ATCC) in USA and Commonwealth Mycological Institute (CMI) in England. In India, the Microbial Type Culture Collection at the Institute of Microbial Technology, Chandigarh and the National Collection of Industrial Microorganisms at the National Chemical Laboratory, Pune are the major sources of authentic fungal cultures useful for projects in industrial microbiology and biotechnology.

Culture collections represent an invaluable infrastructure for aiding advances in microbial biotechnology and serve the following objectives: (1) They conserve under optimum conditions the fungal strains ensuring purity and genetic stability which are most essential for sustainable development; (2) New isolations made from natural resources would be accessioned by a culture collection, identified

properly and conserved optimally in vitro based on a thorough knowledge and experience related to individual strains; (3) Making available the strains maintained in culture collections for exploratory research and development of projects in microbial technology. Effective long-term conservation strategies as well as proper cataloguing of the accessions and follow-up at regular intervals to ensure morphological and physiological stability would be additional responsibilities that culture collections associated with biotechnology product development should undertake.

It would be obvious that contrary to the widely prevalent concept that culture collections represent passive "storehouses" without scope for intellectual activity, they represent a dynamic and vital component in any programme related to biotechnology. The expertise, commitment and technical capability of the staff associated with culture collections play a very important and catalytic role in the success of any biotechnology project. In recent years, the advent of molecular biology techniques have led to the development of fungal strains capable of secreting heterologous proteins. High level of expertise in mycology and molecular biology would be required for the conservation of such strains.

Interest in screening for fungal biodiversity and exploring them for novel metabolites has been on the increase in recent times. Several taxa new to science or those previously not studied intensively for their effective long-term conservation are being discovered in connection with screening programmes. Such cultures would offer opportunities as well as challenges to the mycologists in culture collections with regard to optimising the various parameters essential to ensure long-term conservation with morphological and genetic stability. Optimisation of media composition, pH, temperature of incubation, periodicity of subculture, refrigeration conditions and overall standardising the strategies for successful long-term conservation are some of the most important aspects that are required to be understood through careful scientific investigations, especially in the case of new isolates. For example, species of aspergilli and penicillia

rapidly sporulate on a wide range of synthetic, semisynthetic or natural media within 4–5 days facilitating rapid identification. Others like the pycnidial fungi or ascomycetes may require longer periods of incubation and often may fail to sporulate on conventional sugar-rich mycological media. In these cases, alternate deficient media like Leonian's agar or potato carrot agar may have to be used and optionally by incubating under Near Ultraviolet radiation to promote rapid sporulation. In our studies, a new species of the rare mucoralean fungi, *Benjaminiella multispora* showed very poor sporulation on MGYP or PDA media but the development of sporophores as well as zygospores was rapid and abundant on half strength Emerson's YPSS medium or on a medium containing 5% (w/v) fresh french beans macerated by blending in distilled water and solidified with 2% agar. These examples emphasise the necessity for having a good understanding of the biology and in vitro physiology of new isolates before deciding upon the strategies for their long-term conservation in germplasm banks and culture collections. Thus the most appropriate growth conditions as well as conservation techniques should be established to ensure viability, purity and genetic stability during prolonged conservation. Periodic subculture on agar media is a simple approach to ensure viability of fungal cultures. However the active state of continuous growth gives undesirable opportunities for uncontrolled genetic variations through spontaneous mutations leading to sectoring, pleomorphism etc. and also physiological degeneration through the operation of genetic phenomena like heterokaryosis and parasexual recombinations. It is desirable and even essential that conditions for conservation and storage are selected to minimise the risk from such undesirable changes.

Preservation Techniques

A. Subculturing

Several fungi have been successfully conserved for many years by periodic transfer to suitable agar media. Sometimes it would be advisable to alternate nutritionally rich media with media which are relatively weak in nutrients during successive subcultures. Such a strategy has been recognised as being helpful in maintaining an equilibrium between the physiology of mycelial growth and sporulation in the individual strains. For example, a stock culture on potato dextrose agar or MGYP may be transferred to a weak potato carrot agar or Hay infusion agar in the subsequent transfer followed by its next subculture again on PDA. The choice of the alternative media to be used should be decided only after ensuring that the fungus makes satisfactory growth on either media and recording carefully the growth and sporulation behaviour. In the case of fungi which grow very poorly on weak and nutritionally deficient media, conservation may be done only on the media nutritionally most favourable, and reducing the frequency of subcultures. In our studies, agar media (50 ml) have been taken in 100 ml Erlenmeyer flasks and used as slants for growing and storing fungal cultures. Due to the slow rate of drying of the thickly set agar in the flasks, the viability of the cultures has been observed to be good for periods ranging from six to ten months when stored at 10–15 °C. Since the fungi are strongly aerobic, in our experience, stock cultures are best maintained in wide bored cotton plugged test tubes (19 150 mm). Narrow test tubes with small volume of nutrient agar media in the form of a thin layer are less suited since they promote rapid drying of the cultures. Screw-capped tubes and bottles may be less favourable for some fungi, which are sensitive to the levels of carbon dioxide accumulated during metabolism by the growing culture. For these sensitive strains, the cotton-plugged containers permitting diffusion of gases would be most suitable.

The temperature of conservation or refrigeration temperature of stock cultures also plays an important role in

their survival. Most fungal cultures, especially the sporulating hyphomycetes are compatible with storage temperatures of 4 – 5 °C and readily revive upon subculture. Storage at 0 °C or below of stock cultures on agar has been found to be unsatisfactory for many cultures. Some groups of fungi are very sensitive to storage at 4–5 °C and rapidly lose viability. These include species of *Conidiobolus*, *Cunninghamella* and aquatic zoosporic fungi like *Aphanomyces* and *Pythium*. For such strains it is better to conserve the agar slant stock cultures at 15–18 °C and subculture them at more frequent intervals (say once in 2–3 months). Some zoosporic fungi may require to be subcultured every 40–45 days in order to remain viable. It is very essential to carefully observe that the subcultures do not show any progressive alteration in growth patterns including degree of sporulation. In our experience stock culture agar tubes survive cold storage up to twelve months and should be kept without discarding since they may be used for initiating subcultures in case the serial subcultures have shown pleomorphic or degenerative tendencies.

B. Storage under Mineral Oil

Agar slant cultures are overlaid with sterile liquid paraffin (medicinal grade) which reduces the rapidity of dehydration and also brings down the rate of metabolism by restriction of the oxygen supply. The paraffin oil is sterilised in an autoclave at 121 °C for 60 minutes followed by heating in a hot air oven to clear the opalescence developed during the autoclaving. The depth of the mineral oil overlay above the cultures should not exceed 10 mm since the anaerobic conditions that ensue with deeper layers could lead to the death of the cultures. If the culture is not fully covered by the mineral oil, exposed mycelium or agar on the sides may cause moisture loss leading to drying out of the culture.

While transferring from mineral oil conserved cultures, care must be taken while heating the needle after effecting the transfers. The residual oil will spurt and in the process spores sticking to the needle may get splashed exposing the workers to them. Use of Laminar Flow cabinets with positive pressure

maintained through air screens minimises the risk. While mineral oil overlay is a simple and inexpensive method for prolonging storage life, contamination of the stored cultures has been a problem at times. Also many of the slow growing or feebly sporulating strains either fail to revive or even if revived exhibit loss of sporulation in subsequent subcultures. It is obvious that fungal strains vary widely in their sensitivity to mineral oil conservation and a careful study is essential before deciding upon its suitability for particular strains of fungi. There are however some very satisfactory and successful application of the mineral oil overlay technique for conservation in literature. In a study of long-term storage of wood inhabiting Basidiomycetes, Ascomycetes and Deuteromycetes under mineral oil, **Perrin** (*Mycologia* 71:867–69, 1979) reported viability and revival after 27 years of storage. In other reports, considerable loss of viability after 15 years of storage has been reported, for example in the case of phytopathogenic *Helminthosporium* cultures (**Braverman** & **Crosier**. *Plant Disease Reporter* 50:321–23, 1966). In a comparative study of fungi belonging to diverse taxonomic groups carried out at the Commonwealth Mycological Institute, UK, successful conservation and revival after 12 to 32 years of storage under mineral oil of some of the fungi has been recorded. However it appears that individual strains vary considerably in their response to mineral oil preservation indicating that it is more physiological rather than taxonomic characteristic. Phycomycetous fungi especially the aquatic zoosporic species as well as the saprophytic Entomophthorales respond relatively poorly to mineral oil conservation.

C. Storage in Distilled Water

Sporulating mycelial colonies are cut into blocks, transferred to sterile water, sealed in tubes or bottles and stored at temperatures ranging from 4 to 25 °C. Several strains respond favourably and can be successfully recovered after periods ranging from six months to two years. In our studies, *Conidiobolus coronatus* and *Basidiobolus haptosporus* strains which produced alkaline protease, were successfully revived

after 3–6 months storage at 15–20 °C and conserved the capability to secrete equivalent enzyme levels as those maintained on agar slants. However, recovery and viability were poor in cultures stored in distilled water for more than six months. For hyphomycetous fungi, we have adopted a modified procedure in which the fungi are sporulated on grass leaf placed on 2.5% plain agar plates. Details of this sporulation technique are discussed in the chapter on Physiology of vegetative growth and sporulation. The agar blocks containing the sporulating culture on the grass leaf have been stored at 10–15 °C as well as at room temperature (24–28 °C). Excellent revival without any adverse effect on either viability or sporulation behaviour was observed in species of *Aspergillus, Trichoderma, Volutella* and *Scopulariopsis* when examined after periods ranging from six months to two years. It is noteworthy that during culture on grass leaf, the mycelium penetrates the substomal space through the stomata and ramifies the mesophyll tissue underneath. This dormant mycelium remains viable for long periods and facilitates revival of the culture with minimal alteration in its physiological characteristics. It is often seen that periodic subculture on agar media of many Hyphomycetes lead to degeneration and loss of sporulation especially in the case of phytopathogens like *Curvularia*, *Drechslera* and *Helminthosporium*. Conservation of grass leaf sporulated cultures in sterile distilled water could be a simple and worthwhile technique to be adopted for conservation of their sporulation characters as well as phytopathogenicity.

Successful reports of conservation in sterile distilled water include the study of **McGinnis** et al. (*Appl. Microbiol.* 28:218–22, 1974) who reported survival for 12–60 months of several fungi including dermatophytes and other human pathogenic species. The selection of good sporulating cultures and sufficient inoculum consisting of spores and hyphae suspended in sterile distilled water were the most important factors influencing survival in water over a longer period of time. In a study of 151 wood decaying Basidiomycete cultures **Burdsall and Dorworth** (*Mycologia* 86:275–80, 1994) reported successful storage under sterile distilled water for

seven years. The lack of frequent transfer and the slow growth rate of the cultures under water increased their genetic stability. Those species which could not be lyophilised or preserved under liquid nitrogen could also be stored in sterile distilled water. The cultures were stored in sterile screw-capped plastic cryovials and young mycelial blocks cut out from the growing edge of the colony on 1.5% malt extract agar. Such cultures were most suited for long-term conservation.

D. Storage by Drying

Fungal spores having generally a lower water content than vegetative mycelia are capable of withstanding desiccation. Under dehydrated conditions and water loss, metabolism is slowed down or suspended facilitating survival in a dormant state. Fungal spores are preserved in the dry state by mixing with sterile soil or anhydrous silica gel.

Preservation on Sterile Soil Finely sieved humus-rich soil is transferred to test-tubes, moistened with just sufficient water without excessive flooding and autoclaved at 121 °C for 60 minutes on two successive days. This is followed by heating at 160 °C in a hot air oven until the soil particles become separated and free flowing without clump formation. Sterility check on randomly selected tubes from the lot is performed by transferring aseptically small portions of the sterilised soil into nutrient broth tubes and incubating on the shaker for 72–96 hours. The sterility check is essentially performed to ascertain freedom from aerobic spore forming bacilli, which if present would germinate and show cloudiness of the broth tubes. In that case, the tubes will require to be subjected to further autoclaving and heating. After confirmation of sterility, these soil tubes are ready for use in conservation of the fungal spores.

Spore suspensions of sterile water are added, mixed thoroughly and dried under vacuum.

An alternative and successful method employed in our laboratory is to pour a thin layer of suitable agar medium in a ster-

ile petri plate to obtain a good sporulating colony. Mycelial discs are cut out of the sporulating colony and aseptically added to the sterile soil tubes and kept at room temperature for seven days. During this period, the thin agar layer shows progressive drying and the spores get dispersed in the sterile soil. Soil stock cultures may be conserved in the cold at 5–10 °C. It is generally observed that dry spored hyphomycetes such as *Aspergillus* and *Penicillium* survive well in soil cultures. Thin walled slimy spore masses from genera like *Cephalosporium*, *Myrothecium* or *Volutella* show comparatively poorer survival response. Several of the Mucoralean genera and species of *Conidiobolus* and *Basidiobolus*, characterised by thin walled fragile mycelia and very delicate conidia, fail to survive drying in sterile soil.

Silica Gel Preservation

The silica gel preservation technique involves preparation of spore suspension in autoclaved and cooled (4 °C) skimmed milk. This is then transferred to cold (–20 °C) sterile non-indicator type silica gel beads. The beads are allowed to dry out at room temperature over a period of 10–14 days, when the beads separate easily from one another. Storage is best at 4–7 °C. The culture can be revived by transferring the beads carrying a thin coating of the spores embedded in sterile dried skimmed milk film. Silica gel conservation has been reported satisfactory for many Ascomycetes and Hyphomycetes and Coelomycetes, whereas chytridiomycetes and oomycetes fail to survive under this treatment. Also many zygomycetous fungi with thin walled and rapidly germinating spores are not successfully revived after silica gel drying and storage. In a study of the survival of *Fusarium* species stored on silica gel, **Windels** et al. (*Mycologia* 85:21–23, 1993) reported survival and successful revival of four species after a period of ten years. The degree of survival appeared to be related to the abundance of conidial production in the cultures. Periodic check on viability as well as genetic stability would be advisable for fungal cultures conserved by the silica gel preservation technique.

E. Storage by Freezing

Freezing results in suspension of cellular metabolism. Presently the storage of microorganisms at the ultralow temperature of –196 °C in liquid nitrogen is one of the best and widely applied techniques for long-term conservation of fungal strains. When subjecting fungi to freezing it is very important to suspend them in a medium fortified with cryoprotectants, which aid in preventing intracellular ice crystal formation. These ice crystals, if formed, will damage the cells particularly during the subsequent process of thawing during the culture revival. Dimethylsulfoxide (DMSO), skimmed milk and glycerol are widely used as cryoprotectants and in some cases combination of DMSO and glucose as cryoprotectant has proved useful. The rate of cooling as well as the rate of thawing strongly affect the survival at ultralow temperatures as well as their successful revival. **Smith** et al. (*J. Gen. Microbiol*. 132:2013–21, 1986) in a comparative study of hyphal morphology of *Penicillium expansum* and *Phytophthora nicotianae* during freezing and thawing reported that a slow cooling rate gave highest survival levels. Rapid thawing can be achieved by immersion of the ampoule or storage vial in heated water bath. After the ice melts, the contents are inoculated on a suitable growth medium. Instead of glass vials, polyester films or polypropylene vials are recommended for liquid nitrogen conservation as they eliminate the risk of possible explosion associated with glass vials during exposure to rapid temperature changes. (**Tuite**. *Mycologia* 60:591–94, 1968). Among reports of successful conservation by freezing at ultralow temperatures may be mentioned those of several Ascomycetes, Entomopthorales and Oomycetes for periods varying between two to eighteen years. In all cases mycelia harvested from agar cultures were suspended in cryoprotectant medium and subjected to freezing paying strict attention to the optimal rate of cooling as discussed earlier. Cryopreservation has been found to be favourable for retention of properties after prolonged storage, otherwise lost in continuous subculturing (e.g. loss of pathogenicity in *Phytophthora* (**Dahmen** et al. *Phytopathology* 73:241–46, 1983). Several fungal strains which are sensitive to freeze

drying or lyophilization have been successfully preserved under liquid nitrogen (**Hwang**. *Appl. Microbiol*. 14:784–88, 1966). The only disadvantage of the technique is the high cost of the apparatus and the necessity to have a continuous supply of liquid nitrogen. If liquid nitrogen supply becomes unavailable for some reason, the entire lot of cultures may be lost due to sudden rise in temperature and the subsequent uncontrolled thawing process.

F. Storage by Freeze-drying

Freeze-drying or lyophilisation of cultures involves dehydration of frozen material in an aqueous medium through the sublimation of ice carried out under vacuum. Two basic techniques involved in freeze-drying are (a) the aqueous suspension is frozen by evaporation under reduced pressure or (b) the suspension is frozen prior to evacuation. Freezing prior to evacuation is frequently employed and can be achieved in freeze-drying equipment with shelves in which the rate of cooling can be strictly kept under control. Subsequent drying is carried out under vacuum in vials which are sealed in the chamber while under vacuum. The suspending medium for freeze-drying most widely used is skimmed milk and 5% inositol. Other suspending media employ combinations of skimmed milk, serum, peptone, sugars etc. Freeze-drying is a technique widely applicable to sporulating fungi. However fungi also vary widely in their response and many intrinsic properties associated with individual species with regard to their ability to survive freeze-drying are as yet not fully understood. For example in a study carried out at the Centraalbureau voor Schimmelcultures, Netherlands, the effects of freeze-drying on fungal propagules in relation to the size and wall thickness of the conidia were determined. Species with thick walled and/or large septate conidia dehydrated slowly and consequently tolerated slow cooling only. The composition of the protectant, especially the saccharide component during freeze-drying was found to be a major factor in viability determination (**Tan** & **Stalpers**. *CBS Newsletter*, 18: 8–9, 1994). Freeze-drying is not suitable for

many fungi such as Entomopthorales, oomycetes and mycelial basidiomycete cultures. Generally asporogenous species and those with delicate, thin-walled spores do not survive during freeze-drying. There is considerable strain variation among species and genera of fungi in regard to their relative sensitivity to lyophilisation. It is difficult to predict the survival potential of different strains which can be evaluated only by experimentation. The most critical factors in freeze-drying are (a) composition of the suspending medium (b) cooling rate (c) maintenance of the frozen state during drying (d) residual moisture content after drying and (e) prevention of rehydration and contact with oxygen during processing and storage. It has been found that residual moisture content should not drop below 1% for long-term survival. Freeze-dried ampoule can often be conveniently stored at room temperature without any adverse effects. The process of rehydration for revival also influences survival rates. Rehydration for 30 minutes with sterile distilled water or broth is generally considered adequate. However, for some sensitive fungal cultures rehydration using 0.1% peptone solution for periods up to 24 hours has been recommended.

Freeze-drying offers a suitable technique for long-term conservation without loss in morphological, biochemical and physiological features. There is no risk of contamination in sealed vial during storage. It is a safe and recommended method of long-term conservation for all those strains which are not sensitive to the lyophilisation procedure.

Role of Culture Collections in an Era of Biotechnology

Culture collections can be regarded as the golden link between biodiversity exploration and biotechnology applications. They play a vital role in making available reliable and authenticated microbial cultures for biotechnology research and development. Collection, characterisation and conservation are

integral components of the overall process of screening microbial cultures for novel and useful bioactive metabolites.

Culture collections of a specialised nature are often built around the expertise and research interests of an individual research scientist. Monographic studies on individual genera and related taxonomic groups result in collection of authenticated species which would serve as reference standards for future basic research studies or contribute valuable germplasm for biotechnology explorations. Often such collections vanish once the monograph is published and when the research scientist's interests shift to other projects. In the interest of conserving biodiversity for biotechnology progress, it would be obligatory for the strains to be conserved in a well-recognised culture collection and for which the expert scientist should give the full benefit of his expertise with regard to the protocols standardised for their long-term conservation.

Culture collections serve as important repositories for microbial cultures on which patent applications have been filed. In biotechnological investigations, which involves the use of new microorganisms, i.e. those not generally known or not readily isolated or possessing special attributes, a simple written description is not considered sufficient for the purpose of a patent disclosure. In spite of providing the most detailed description, the strain itself must be available for "anyone skilled in the art" to verify and reproduce the claims made in the patent application. Thus the strain itself forms the most vital component of a biotechnology patent disclosure. In USA and Europe, patenting of biotechnological inventions require that a culture of the microorganism used is deposited in a recognised culture collection so that a ready supply of the microorganism is available at some stages of the patenting procedure. The American Type Culture Collection (ATCC) was the first culture collection in the USA and apparently the first in the world to access a patent strain.

Subsequently, with many developed countries contributing to biotechnological process development and also for simplifying the international patenting of biotechnology

processes, the Patent Cooperative Treaty (PCT) was signed in 1970, by all the industrially developed countries. The clauses of the PCT treaty were further amended in 1978 and 1979. It allows an applicant to file a single application in a standard format through the applicant's national patent office and have the application recognised as a valid filing in as many PCT countries as selected. Seeking patent protection in several countries under PCT necessitated multiple deposits of microbial cultures. To overcome this, the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for patent procedure was signed on April 28, 1977, and came into force on August 19, 1980. Under the provisions of this treaty, a single culture deposition with a recognized depository authority would satisfy the requirement of all the countries selected in a multicountry patent filing under the PCT. The ATCC was the first International Depository Authority (IDA) approved by the World Intellectual Property Organization (WIPO) under the Budapest treaty.

In order to qualify for an IDA status, a culture collection in a member country must comply with the requirements stipulated under the Budapest treaty. These include technical and administrative competence, and agreement of the depository with the terms and conditions applicable to the various IDAs worldwide. The facilities must meet the international standards for accepting, maintaining and preserving the patent strains and have high level professional competence as well as financial stability. These conditions are very important since under the Budapest treaty, the patent culture must be maintained for thirty years.

India became a party to the Budapest treaty on the International Recognition of the Deposit of Microorganisms for patent procedure only on December 17, 2001 (Intellectual Property Rights (IPR), TIFAC Bulletin 8:1, Jan–Feb 2002). Presently 54 countries are members of this treaty. However not all countries have their own recognized IDAs. Presently 20 countries have 33 IDAs as follows: U.S.A. (2), U.K. (6), Russian Federation (3), South Korea (3), China (2), Italy (2) and other countries have the remaining 15 IDAs. Most Asian, African

and South American countries have not so far been signatories for the Budapest treaty. For example, before filing a U.S. Patent application, the strain on which the patent application is based has to be deposited with the ATCC and the ATCC accession number has to be cited in the U.S. Patent application that is being filed. It is obvious that the importance of culture collections is becoming greater as biotechnological advances are made and this activity needs to be taken up with much greater seriousness in developing countries like India with a high level of intellectual potential. The Microbial Type Culture Collection (MTCC) at IMTECH, Chandigarh, India has recently been granted IDA recognition for patent purposes. **Stevenson** & **Jong** (*World J. Microbiol. Biotechnol.* 8:229–235, 1992) have emphasised that because of recent advances in biotechnology, culture collections face increased demands not only for quality cultures but also current information. The necessity for subjecting culture collections to Good Laboratory Practice (GLP) guidelines would be beneficial in assuring quality with regard to management, technical personnel and quality assurance. In the context of global competitiveness, it would be mandatory for countries of the developing world to ensure that their biotechnology programmes have the strong foundation and support from national culture collections conforming with the high standards established in the culture collections of the developed world.

World Federation of Culture Collections

The growing importance of microbial cultures in industrial microbiology and biotechnology led to the establishment of the World Federation of Culture Collections (WFCC) as a federation under the International Union of Microbiological Societies (IUMS) with responsibility for the promotion and development of collections of cultures of microorganisms and cultured cells. Truly, the increasing demands on culture collections for authenticated and reliable biological material and associated formation have paralleled the growth of biotechnology. The WFCC is an international body and has

published in 1999 guidelines for the establishment and operation of collections of cultures of microorganisms. Copies of the guidelines are obtainable from Dr Alan Doyle, Secretary WFCC, The Wellcome Trust, 183, Euston Road, London NW12BE, United Kingdom.

CHAPTER 4

Physiology

Vegetative Growth and Reproduction

Introduction

Fungi display a diversity of morphological forms and structural complexity ranging from unicellular chytridiomycetes and yeasts to multicellular filamentous mycelial forms and complex fruit bodies of macrofungi like mushrooms and polypores. The hypha is the essential unit of vegetative structure in the filamentous fungi collectively referred to as the mycelium. Hyphae present a large surface area through which exchange of materials with the environment is greatly facilitated. Nutrients essential for the biosynthetic processes of growth and development are absorbed, while excretion of products of catabolism takes place. The vegetative phase of growth is followed by a reproductive phase, which in most cases leads to the formation of spores—asexual or sexual or both types. The spores are propagules which get dispersed and germinate on fresh substrates under favourable conditions to give rise to vegetative growth of a new individual of the species.

Ecologically, fungi represent a very successful group of heterotrophs capable of colonising a wide range of natural substrates. Their range of metabolic diversity and adaptability to utilise diverse nutrient substrates through their wide range of enzyme systems make the fungi extremely interesting and potentially versatile and invaluable for

biotechnological applications. That the fungi represent a group nutritionally distinct from plants and animals was recognised and has been discussed by **Whittaker** (*Science* 163:150–60, 1969). While plants use radiant energy and animals engulf their food, fungi absorb their nutrients from the environment in a heterotrophic mode of nutrition, in which secretory enzymes play a significant role in the external digestion of natural substrates which are complex in their chemical structures. The potential of fungi as agents in the recycling of a wide range of organic substrates including recalcitrant natural and man-made compounds make them very important and interesting both academically and technologically.

The Fungal Spore

The fungal spore represents a specialised structure designated by the species for its survival and dispersal. Spores are generally characterised by low metabolic activity, low water content and minimal metabolic turnover. Asexual spores produced by hyphomycetous fungi such as *Aspergillus* and *Penicillium* are very useful in conservation in pure culture, industrial production strains in the laboratory for various manufacturing processes. These conidiospores readily germinate within a few hours upon transfer to fresh media and initiate the process of vegetative development. Many sexually formed spores (e.g. zygospores of Mucorales, ascospores of ascomycetous fungi like *Neurospora*, basidiospores of mushrooms, rust teleutospores, smut chlamydospores etc.) will not readily germinate but require a period of dormancy and rest before they are capable of germination. Such behaviour enables the species to survive the adverse environmental conditions. Many of the spores with a "constitutive dormancy" can be activated to germinate overcoming the innate barriers such as the barrier against penetration of nutrients or presence of a metabolic block or self-inhibitor. Heat inactivation, treatment with chemicals or appropriate enzymes have been successfully employed to

break the dormancy and achieve germination in the case of several dormant ascospores and basidiospores.

Fungal spores are aerobic and as such there is an obligate requirement for oxygen. Some spores require carbon dioxide. While an external carbohydrate source in the germination medium is helpful for germination, many of the spores and especially those rich in endogenous nutrient reserves are capable of germination even in simple distilled water or nutrient salt solution in the absence of added carbohydrates. During the germination of fungal spores many processes and changes take place resuming growth and development from a resting structure in which a morphologically different form results. From an essentially nonpolar spore, a polar germ tube emerges which continues growth by extension from the tip. In the developmental cycle of fungi, the spore can be regarded as the beginning as well as the end to the growth process. Physiological changes associated with the germination process in dormant spores include increase in endoplasmic reticulum, mitochondrial numbers and development of wall layers in the interior regions leading to a noticeable swelling in spore size prior to the onset of germination. Growth polarity is established and the filamentous hypha emerges out in which the cell wall is continuous with the innermost layer of the spore wall.

The Vegetative Development

A. The Cell Wall

The presence of a rigid cell wall to a large extent determines the cellular form of fungi. Fungal cell wall is a dynamic complex structure and is the location of various enzymic activities. It is intimately involved in and responsible for fungal morphogenesis. Studies on the chemical composition of cell walls have provided information on the nature of its macromolecular constituents, while data on their spatial arrangements has been obtained from electron micrographs of

cell wall material. Chitin and cellulose constitute the macromolecular constituents of cell walls occurring along with other polysaccharides, proteins and lipids. Microfibrils of chitin and cellulose are embedded in a polysaccharide-rich matrix, which contributes to a major dry weight of the fungal cell wall. The major building units for microfibril synthesis are glucose and *N*-acetyl glucosamine.

In the multicellular fungi, the hypha represents the fundamental growth unit, which may or may not be divided into cells by septa formation. The hypha is a tubular structure enclosing all the cytoplasmic components of an eukaryotic cell, with a rigid cell wall. Growth of hyphae is highly polarised and takes place at one end. Cell wall material is laid such that the filamentous shape is retained during the growth phase. Hyphal elongation is facilitated by apical deposition of wall skeletal polysaccharides especially chitin and β -glucan. In the apical region, the wall is plastic and allows insertion of new material and progressively undergoes rigidification to become the lateral wall of the hyphae. Rigidification of the newly formed wall is effected by covalent cross-linking of wall materials, especially chitin and β (1–3) glucans and hydrogen bonding of adjacent polysaccharide chains, especially chitin to form microfibrils. Recent studies have provided evidence that mannoproteins are deposited during maturation of the wall. These are synthesised by the involvement of endoplasmic reticulum and golgi bodies and secreted into the developing wall by exocytosis. Fungal cell wall organisation is a complex and elaborate process which includes coordinated interrelationships between the enzymatic steps leading to formation of cell wall precursor skeletons, their transportation and assemblage to result in the three-dimensional hyphal form.

B. Hyphal growth

In the filamentous fungi, microscopic strands termed hyphae constitute the vegetative state. Active growth in length of hyphae takes place in their apices while branching takes place behind the growing tip. The thallus constituted by the mass of

vegetative hyphae is termed mycelium. The cytoplasm is continuous in non-septate vegetative mycelia (e.g. Mucorales, aquatic phycomycetes) while in most fungi the mycelium is septate but the cytoplasmic continuity is maintained through septal pores between cells of the multicellular hyphae. Even in species with non-septate coenocytic mycelia, septa are formed segregating older parts or during the differentiation of reproductive parts. In the basidiomycetous fungi, the pores in the centre of the septum are surrounded by an annular thickening at their edges referred to as "dolipores". The nuclei of fungi are typically eukaryotic and possess a nuclear membrane, nucleolus and chromosomes. In coenocytic mycelia, the nuclei are randomly embedded in the cytoplasm while in the septate hyphae individual cells are uni- or multinucleate.

Recent studies have established that apical growth of fungal hyphae is closely associated with the forward transport of a range of vesicles which provide new cell membrane material. Some vesicles carry membrane bound enzymes (e.g. chitosomes carrying the zymogenic chitin synthase) while others transport material for secretions such as wall mannoproteins and extracellular enzymes. A germinating spore produces one or more germtubes and exponential growth of a fungal colony is achieved by the formation of sub-apical branches, each of which becomes an apically elongated hypha. The hyphae in young colonies have been regarded by many workers as juvenile which undergo slow maturation in slow-growing colonies. The most characteristic shape determining component of fungal walls is chitin (β-1, 4 linked homopolymer of *N*-acetylglucosamine). Apical extension of hyphae involves localised deposition of newly synthesised wall skeletal material, especially chitin. In locations where chitin deposition occurs in the lateral wall, a new branch formation takes place. Chitin synthesis thus plays a key role in the hyphal growth and differentiation. Also the involvement of lytic enzymes in apical growth of hyphae has been considered important and recent concepts recognise cell wall growth as involving a dynamic balance between enzymes lysing walls and those synthesising them.

C. Colony Growth

As already stated, in a growing colony the mycelium is composed of hyphae which are best regarded as similar and functionally inter-related elements. Individual hyphae establish interconnections with adjacent hyphae through anastomosis at an early stage. This enables communication as well as passage of cytoplasm, food and genetic materials from one mycelium to another. Different degrees of coordination among the hyphae takes place during the process of growth and differentiation in the growing colony. During surface growth on solid media such as agar plates, the growth of the colony resulting in radial spread is essentially as a result of the apical growth process in the growing mycelia. Obviously it leads to a heterogenous mycelial biomass differing not only in age but also in its state of physiological development and differentiation. Different fungal colonies exhibit varying levels of development of aerial mycelium, while the substrate mycelium, depending on the culture medium and growth conditions, may exhibit localisation of contents and wall thickening leading to chlamydospore formation in the older regions. In some of the special cases such as *Sclerotium rolfsii*, maturing vegetative mycelium undergoes differentiation resulting in the formation of discrete horny sclerotial bodies as a result of aggregation of the hyphal mass surrounded by a thick and horny wall material. Sclerotial formation of a similar nature is observed in the case of *Claviceps*, the commercially important source of ergot alkaloids. The ergot sclerotia form on the inflorescence of the grass hosts such as rye and later in the season germinate to produce perithecia and ascospores of the fungus under favourable conditions to infect fresh host material.

In a fungal colony growing on a solid substrate the most significant factor related to the heterogeneity is the onset of sporulation. In the older regions or under conditions leading to unfavourable situations for continued vegetative growth, sporulation is triggered. For example, in agar slants many fungi show sporulation earlier on the drying edge of the agar slants whereas in the central regions where the media layer is

thick and moisture levels are high, sporulation takes place much later and thus there is a physiological age difference in the spore populations in fungal cultures growing on agar slants. Fungi like *Aspergillus, Penicillium* and *Mucor*, widely employed in fermentation studies are capable of ready and ample sporulation on various nutrient media, whereas in many other slower growing and weakly sporulating species, special conditions may be required for inducing sporulation in the growing colonies. Ascomycetous fungi as well as pycnidia forming coelomycetes often take more than two to three weeks before sporulation is even initiated and that too on weak nutrient media such as potato carrot agar or Leonian's medium. Media rich in sugars and organic nitrogen supplements often support prolonged vegetative growth and considerable delay is observed in triggering sporulation in these cultures. In some instances such rich media may not support sporulation at all.

Near Ultra-violet radiation (Black Light) has been studied for its efficacy in enhancing rapid sporulation of several fungi in the growing colonies. **Leach** (*Can. J. Bot.* 40:151–61, 1962) observed that sporulation of 34 species of fungi was most effectively induced by exposure to Near Ultra-Violet radiation (NUV) and that long exposures were neither lethal nor inhibitory. Stimulation for sporulation occurred regardless of the many other environmental factors. NUV radiation at 3100 to 4100 A° (maximal at 3650 A°) was stimulatory to sporulation particularly in the hyphomycetes and several mitosporic fungi. In the absence of "Black lamps" cool white fluorescent tubes which emit significant amounts of NUV radiation can be used as an alternative. An alternating cycle of 12 hours of radiation followed by 12 hours of incubation in the dark may be planned, particularly for some fungi which have an obligatory requirement for growth in the dark after a period of irradiation. Irradiation should commence after 3–4 days of incubation when sufficient mycelial growth has taken place. It is preferable to carry out the studies in plastic petri dishes which effectively transmit the NUV rays whereas glass dishes are impervious to these rays and as such will not serve the purpose for induction of sporulation. **Leach.** (*Trans. Brit.*

Mycol. Soc. 47:153, 1964) postulated that photo-induced asexual sporulation in many fungi involves the same mechanism. The radiant energy is captured by a receptor pigment which absorbs UV radiation much more effectively. Isolations made from a wide range of fungi contain a substance having maximum absorption at 310 nm (designated P310) in the water extracts of UV treated mycelium but such a substance is absent or occurs in very low amounts in extracts from dark grown cells (**Leach**. *Can. J. Bot*. 43:185–200, 1965). It has been suggested that P310 is an essential compound which can be synthesised by some fungi in the dark on a rich medium, but on an incomplete medium an important photochemical reaction is necessary to induce its formation. Among basidiomycetes, light may be necessary for initiating the primordia of fruiting structures or it may affect the subsequent stages of development such as stipe elongation, pileus formation or even hymenium and spore maturation. In mycology, the basis for taxonomy and classification is based on the morphology of spores and the manner in which the spores are produced by the vegetative mycelia. Fungi which do not form spores even after prolonged incubation and the remaining mycelial forms have been classified under Mycelia Sterilia or Aganomycetes.

Grass Leaf Sporulation Technique for Inducing Sporulation

(**Srinivasan** et al. *Trans. Brit. Mycol. Soc*. 86:31–35, 1971)

The technique originally developed for rapid sporulation and conservation of seed-borne fungi has been subsequently found applicable to a wide range of sporulating hyphomycetes and coelomycetes. It has provided scope for quick identification of fungal colonies encountered on isolation plates during screening. It has to be tried out for diverse fungal isolates before its full application potential can be judged.

The technique consists of transferring to the surface of sterile hard agar—(3%) plain agar—autoclaved bits of grass leaf such as bajra (*Pennisetum glaucum)* leaves ensuring that the agar as well as the grass leaf surface are dry and free from moisture films. Mycelial transfers from young fungal colonies are laid on the grass leaf bits with part of the transfer resting

on the agar surface. The plates are preferably incubated in chambers to provide 12-hour radiation from NUV light (using plastic culture plates) or alternatively on laboratory benches exposed to day-light or fluorescent tube-light illumination for 8–12 hours. In hyphomycetous fungi like *Curvularia, Dreschlera, Aspergillus, Penicillium* and *Trichoderma*, the mycelial growth originating from the inoculum quickly penetrates through the stomata. Due to the restricted nutrients available the sporulating structures are soon seen and within 4–5 days the surface of the grass leaf is covered with numerous sporulating structures typical of the species. The growing mycelium is essentially confined to the grass mesophyll in the substomal region. In the case of pycnidial fungi like *Phoma* and *Ciliochorella*, formation and maturation of the fruiting structures may take up to two to three weeks. Sporodochial fungi such as *Volutella*, *Myrothecium* etc. form the typical sporulating structures within 7–10 days. The technique facilitates detailed morphological studies on the sporulating structures under the microscope in situ without any interference from the mycelial growth. Permanent slides preparation as well as taking photomicrographs to bring out the distinguishing morphological features are made easy by this technique. The viability of grass leaf culture is good even after prolonged incubation during which time the surface-borne spores as well as the agar may show considerable dehydration and drying. Addition of sterile water to the drying agar slopes to moisten the grass leaf followed by transfer to fresh media readily revives the culture growing out of the hibernated mycelium from within the leaf tissue.

In our experience the technique appears to be particularly well suited for lignocellulose-degrading fungi, many of which show decline in sporulation behaviour after prolonged subculturing on conventional sugar-rich mycological media. Some of these are also sensitive to lyophilisation and the cultures revived out of the lyophils either show poor viability or loss of sporulation ability. The preservation technique closely simulates the manner in which the strains survive in the natural environment and the degree of variants arising from the cultures revived in regard to morphological features

as well as ability to secrete extracellular enzymes have been found to be minimal. Industrial biotechnologists may find this technique easy and very practical for identification and long-term conservation of their isolates.

D. Morphogenesis of Vegetative Hyphae: Dimorphism

Fungal dimorphism is expressed in cellular terms by the ability of a fungal strain to grow predominantly either as hyphae (characterised by linear or apical growth) or yeast-like spherical or ovoid cells. Yeast-mycelial dimorphism pertains to the morphological and macroscopic differences observed in agar or liquid cultures which are amenable to control by experimental conditions. Many human pathogens involved in deep mycoses like *Blastomyces* and *Histoplasma* exhibit dimorphism, appearing yeast-like in the infective phase while exhibiting mycelial form when cultivated on conventional mycological media such as Sabourad's agar.

Understanding the morphogenetic events involved in dimorphism and correlating them with physiological, biochemical and molecular events underlying this transition from one phase to the other would be invaluable in getting a better insight into the pathogenic behaviour of these fungi. Many mucoralean fungi, which are non-pathogenic such as *Mucor racemosus, M. rouxii, Benjaminiella poitrasii* and *Mycotypha* sp. exhibit dimorphism which is related to the cultural environment and these fungi have been studied intensively from the academic point of view to generate basic data and knowledge regarding the phenomenon. It is especially interesting that dimorphism is reversible from one phase to the other. Such studies have attempted to understand as to why the hyphal form has its growth restricted to the apical region, whereas under the spherical growth pattern of the yeast phase, the growth is throughout generalised.

Nutritional factors exercising control on dimorphism have been studied with Mucoralean fungi such as *Mucor*, *Mycotypha* etc. The influence of hexose concentration and carbon dioxide tension on the morphogenesis of *Mucor* has

been well documented (**Bartnicki-Garcia**. *J. Bact*. 96:1586 –94, 1968). *M. rouxii* shows exclusively yeast-like growth under anaerobic conditions or with a carbon dioxide content of 30%. The concentration and type of hexose in the medium are also main factors in dimorphism. For example, *Benjaminiella* (Cokeromyces) *poitrasii* grown on agar media containing 25% glucose appears yeast like with total suppression of asexual and sexual sporogenesis. (**Srinivasan** et al. *Current Sci*. 55:493–97, 1986). In *Mucor* at 30% carbon dioxide atmosphere growth was mycelial when the hexose concentration was 0.05%, while at 50% or more hexose concentration, the yeast form developed.

The presence of chemical agents such as phenethyl alcohol stimulates alcohol fermentation and inhibits oxidative phosphorylation inducing the formation of yeast-like cells. Involvement of mitochondrial activity in the morphogenetic process of *M. rouxii* has been demonstrated by germinating its spores aerobically in presence of chloramphenicol. The antibiotic induces yeast growth—an effect which is potentiated if the glucose in the medium is raised to 5%. In *Mycotypha* also chloramphenicol stimulated yeast-like morphogenesis. Agents blocking the electron transport chain or its generation such as rotanone, antimycin A, KCN, oligomycin, amytal, thenoyltrifluoro acetone and 2, 4 dinitrophenol caused suppression of mycelial form and favoured yeast growth (**Schulz** et al. *J. general. Microbiol*. 82:1–13, 1976).

The problem of dimorphism in various fungi is a complex one and often not amenable to be explained in simple cause and effect terms. While many enzyme activities and physiological processes alter during the dimorphic change, none seems to be causal. Cyclic AMP and other signalling molecules as well as enzymes involved in regulating their intracellular concentrations have been shown to exhibit consistent dimorphism related patterns of change. (**Orlowski**. *Can. J. Bot*. 73:S326–S334, 1995). Metabolic changes accompany the yeast-mycelial transition in pathogenic fungi. In *Histoplasma capsulatum* and *Paracoccidioides brasiliensis*, the Y-form have a more active tricarboxylic acid cycle than the

M-form. Mannan which is absent in most filamentous fungi is present up to six times more in yeast phase cell wall compared to cell wall of filamentous phase. Growth polarisation is the crucial difference between M and Y forms. Dimorphism is affected by environmental factors such as temperature, sulphydryl compounds and aeration. Development of Y-phase is consequential to selective inhibition or interference with morphological mechanisms indispensable for cylindrical cell formation.

An interesting observation related to dimorphism in the mucoralean fungi which may have some biotechnological significance is that the yeast-phase cells ferment sugars to produce alcohol. This alcoholic fermentation can be interrupted by addition of benzaldehyde and through the action of the carboligase enzyme present in the yeast-phase cells, bring about the formation of phenylacetylcarbinol, the key intermediate for L-ephedrine production by fermentation. Presently yeast cells (*Saccharomyces cerevisiae*) are employed for bringing about this reaction, but the possibility of using the fungal yeast-phase cells may also deserve further investigation for technological possibilities.

Asexual Reproduction

The diversity of spore forms that the fungi produce and the manner in which they are formed contribute essentially towards the taxonomy, classification and identification of fungi based on their morphological features. The asexual spores are the principal agents of dispersal and species propagation, while the sexual spores are the products of genetic recombination and most often function as dormant phases in the life cycle to tide over adverse environmental factors. The zoospore forming aquatic fungi range from unicellular members like *Olpidium* in which the entire thallus gets converted into a sporangium and its contents undergo division to differentiate into zoospores to the filamentous forms like the Saprolegniales in which well-differentiated

sporangia are formed and zoospores are released at maturity, often through special releasing mechanisms. In the zygomycetous fungi belonging to the Mucorales, the asexual spores are differentiated in well-defined sporangia as in *Mucor* or in noncolumellate sporangiola as in *Helicostylum* or *Benjaminiella* multispora or in cases like *Cunninghamella* the spores are borne externally on the vesicles terminating aerial conidiophores. In the other group of zygomycetous fungi, viz. Entomophthorales represented by *Conidiobolus*, asexual reproduction takes place by the formation of globose conidia terminating unbranched conidiophores from which the spores are forcibly discharged at maturity. These discharged spores either germinate by germtube formation or give rise to secondary spores by evagination of their contents or in some species like *C. coronatus* may give rise to a number of small microconidia developed on radial sterigmata.

In the mitosporic fungi belonging to the hyphomycetes, asexual conidia are developed on conidiophores of varied complexity. These range from simple unbranched or branched extensions of the vegetative mycelium to well-organized conidiophores with vesicles, sterigmata, phialides etc. as in the important genera like *Aspergillus*, *Penicillium*, *Paecilomyces*, etc. Taxonomy of the hyphomycetous fungi is centred around the morphological features of the conidiophores as well as the characteristic features of the conidia. In the coelomycetes or pycnidia-forming fungi as well as those in which the spores are aggregated in well-defined sporodochia, their taxonomy also takes into account the morphological features of these spore bearing structures for the purpose of identification and classification. The aquatic hyphomycetes inhabiting fresh water streams and foams in running water sources possess various types of appendages helping them to achieve buoyancy and their classification is essentially based on the morphological features of their spore appendages.

In fungi, differentiation of spores commonly takes place after the phase of rapid vegetative growth. Alternative metabolic pathways are involved in the processes associated with vegetative growth and cell division on the one hand and

morphogenesis and spore differentiation on the other. As early as 1898, **Klebs** postulated that growth and sporulation are incompatible and that reproduction is initiated by factors which check or retard growth. **Hawker** in her extensive studies on the effects of environmental factors on reproduction came to the conclusion that conditions permitting spore formation are mostly of a narrower range than those permitting vegetative growth. (**L.E. Hawker**. *The Physiology of Reproduction in Fungi*, Cambridge University Press, 1957).

Genetic control of conidiospore formation has been studied in *Aspergillus nidulans*. Some of the key findings can be summarised as follows: Conidiation does not take place until the cells have gone through a defined period of vegetative growth. Synchronous developmental induction of sporulation has been studied by harvesting mycelia grown in submerged culture and then exposing them to air. By varying the length of time that the hyphal cultures are maintained in the submerged cultures, it has been shown that there is a minimum growth period before cells respond to induction. Cells require approximately 18 hours of growth before they are ready to respond to the inductive signals provided by exposure to air. Accordingly it is interpreted that the initial conidiophore development in *A. nidulans* occurs as an integral part of the life cycle rather than as a response to unfavourable environmental conditions. Differentiation of the multiple cell types in *A. nidulans* conidiophore is correlated with activation of several hundred genes and to date only a small number of genes have been analysed for their function. Studies by **Timberlake** have shown that approximately 1200 diverse mRNAs accumulate in varying concentrations specifically during conidiation. Spore wall proteins encoding genes have also been investigated and genes such as rodA and dewA have been characterised, which encode proteins that contribute to the hydrophobicity of conidia and presumably facilitate their dispersal by air. Both of these genes were found to belong to a class of proteins found in fungi which have been termed Hydrophobins.

Hydrophobins

Hydrophobins are proteins which play an important role in releasing the fungal structures from their moist and damp environment and facilitate them to grow into the air and form spores. Fungal hydrophobins have been identified in Ascomycetes and Basidiomycetes (and their anamorphic states). These are structural components found in the spore walls as well as in aerial hyphae and contribute to the hydrophobicity of these structures. The habitat in which fungi live and flourish is mostly moist and humid such as wood, leaf litter or in the case of pathogenic species plant or animal tissues. In order to propagate and disperse their spores, fungi need to escape their moist surroundings and push their sporulating structures into the air. For a fungus to produce such aerial structures it has to overcome and escape the surface tension of the water film surrounding the fungal colony. This is accomplished by the action of a remarkable group of proteins termed hydrophobins. These are small proteins with 96 to 187 amino acids. They all contain eight cysteine residues arranged in a conserved pattern but differ much in their amino acid composition. In spite of their diversity even quite different hydrophobins are functionally interchangeable among species, suggesting that they also share conserved physical characteristics. (**Williams**. *Nature* 394:55–57, 1998). The most extensively studied hydrophobin is SC3, a hydrophobin produced by the basidiomycete *Schizophyllum commune*. SC3 forms a water repellant (hydrophobic) outer coating on aerial hyphae which can be observed under the electron microscope as a layer of interwoven rodlets. SC3 is secreted as a monomer but when it encounters an air-water interface or an interface with a hydrophobic surface it aggregates into large polymeric complex. Structural alterations in SC3 take place in response to changing environmental conditions encountered by the fungus as it grows from the water into the air allowing the fungus to produce hydrophobic aerial hyphae. Our knowledge of fungal hydrophobins is far from complete and future researches may throw additional significant light on their roles in fungal life cycle. For example a variety of

hydrophobins with diverse biochemical properties have been discovered in fungi, possibly indicating their role in other developmental processes besides their role in aerial growth facilitation. (**Talbot**. *Nature* 398:295–96, 1999). An exhaustive review on hydrophobins has been published by **Wessels** (*Adv. Microbiol Physiol*. 38:1–45, 1997), which may be consulted for greater details on hydrophobins.

Sexual Reproduction

Besides asexual spore formation, sexual reproduction is widely prevalent in diverse groups of fungi. Sexual reproduction involves the union of two compatible nuclei after plasmogamy of the gametes or gametangia bring them together in one cell and karyogamy unites them into one diploid nucleus. Genetic recombination in the diploid nuclei and subsequently meiosis to restore the haploid state are the ultimate stages of sexual reproduction. During this process an opportunity is provided for genetic recombination and segregation of characters and is helpful in the evolution of newer variants adapted for better survival in the natural environment. Besides, the products of sexual reproduction such as zygospores, ascospores and basidiospores have a vital role in conserving the species by virtue of their ability to remain dormant and tide over the unfavourable environmental conditions.

Sexuality is more prominent in the aquatic zoosporic mycelial fungi in which well-defined oogonia and antheridia develop on the same (homothallic) or different (heterothallic) thalli and the fusion of the male gamete (antherozooid) with the female gamete (egg cell) is mediated by secretion of sex attractant hormones in these fungi such as *Allomyces*. In the zygomycetes represented by the Mucorales and Entomophthorales, there are no gametes but gametangia are differentiated from the mycelia which undergo fusion and the fusion product develops the characteristic zygospores, often with very distinguishing morphological features.

In Ascomycetes and Basidiomycetes, plasmogamy and karyogamy may be separated in time and space and the dikaryotic condition may be perpetuated from cell to cell by conjugate or by simultaneous division of the two associated haploid nuclei. Fusion of the two haploid nuclei takes place later and the formation of the products of sexual reproduction, ascospores and basidiospores takes place within elaborate fruit bodies such as perithecia and basidiocarps respectively.

For many of the conidial fungi the sexual stage is not known and they have been grouped together under Deuteromycetes or fungi imperfecti merely as a working classification without any distinctive taxonomic status. In mycological literature it is customary to refer to the sexual stage as the "perfect" stage while the asexual conidial stage is referred to as the "imperfect" stage. Technically these are also referred to as "teleomorph" and "anamorph" respectively. Often through cultural studies conidial and sexual stages have been bridged and in such cases the fungus nomenclature is primarily designated on the basis of the perfect or the teleomorphic stage. For example *Neurospora crassa* is a heterothallic fungus which will form the sexual stage only when the appropriate opposite mating types are allowed to cross under suitable cultivation conditions. However in the cultures used in biotechnology, what is present is generally only the conidial or anamorphic state referable to the genus *Monilia* with orange coloured chains of conidia (*M. crassa*). Similarly *Gibberella fujikuroi* is the perfect stage of the gibberellic acid producing ascomycete fungus whose conidial state is *Fusarium moniliforme*. The white rot fungus *Sporotrichum pulverulentum* secreting cellulase as well as ligninase was designated after its basidial state was discovered by the name *Phanerochaete chrysosporium* with *S. pulverulentum* as its conidial or anamorphic stage.

To the industrial biotechnologists not fully familiar with rules of mycological nomenclature, these dual names for the same fungus are sure to create considerable confusion. However a little effort to understand the principles behind the use of these names will to a large extent lessen the confusion. In sexually reproducing organisms, the products of sexual

reproduction would be expected to exhibit higher levels of genetic variation due to the accompanying process of genetic recombination taking place during their formation. This could have adverse effects on the genetic stability as well as the level of production of desired metabolites in fermentation processes. Even in the case of fungal strains with a known perfect state, the strains employed for fermentation, mutation for overproduction etc. are carried out only on the conidial stage since genetic stability will not be affected in these conidial strains due to the factor of recombination associated with the sexual stage. In a few instances of industrial strains like *Chaetomium*, the only spore form known are the ascospores borne inside asci developed within characteristic perithecia. Formation and maturation of these perithecia takes up to two weeks or more and so fermentation studies are generally initiated with mycelia taken from young colonies while the parent stocks with mature perithecia and ascospores are suitably cryopreserved to minimise genetic variations accompanying frequent subcultures.

The anamorph–teleomorph connection of fungal strains is a topic of great fundamental interest in mycology. In several instances diverse teleomorphs may have the same anamorph as in *Fusarium*. Here the conidial stage is accompanied by different ascomycetous genera like *Gibberella, Nectria, Neocosmospora* etc. *Aspergillus* and *Penicillium* species have perfect stages referable to the ascomycetous genera *Eurotium*, *Talaromyces* etc. The ubiquitous *Aspergillus niger*—one of the most industrially useful species—is known only in its anamorphic (conidial) state and its perfect state is unknown. Similarly there is no perfect state known for the penicillin producing *Penicillium chrysogenum*.

Hormonal Control of Sexual Reproduction

In several fungi, secretion of sexual hormones which control and direct the initiation of sexuality has been well established.

The phenomenon of hormonal control in aquatic zoosporic fungi has been studied in detail and with interesting findings. In *Allomyces*, sexual reproduction is manifested by the copulation of free swimming anisogamous planogametes in an ambient liquid medium. The male gametes are much smaller than the female gametes, which release a sperm attractant designated as Sirenin prior to their release from the gametangium. Sirenin has been shown to be an oxidative sesquiterpene and is active at concentrations as low as 10

type may regularly produce a diffusible substance (progamone) that is converted to the hormone (gamone) by the other strain. In fungi like *Blakeslea trispora* accumulation of β-carotene accompanies sexual reproduction and has been regarded as a possible secondary effect following the onset of sexual reproduction (**van Den Ende**. *J. Bact*. 96:1298–1303, 1968).

Sterols have a significant role to play in fungal life cycles. In the oomycetes, sterols have been shown to be required for sexual reproduction and are also needed for normal production of the sporangia and zoospores. Cholesterol or closely related sterols appear to be essential for sexual reproduction, although this effect can be nullified by the presence of acidic amino acids (**Haskins** et al. *Can. J. Microbiol*. 10:187–95, 1964, **Seitsma & Haskins**. *Can. J. Microbiol*. 13:361–67, 1967). In *Sordaria fimicola*, an ascomycete, the addition of cholesterol to the culture medium promoted the extent of perithecial development.

Instances of secondary metabolite accumulation accompanying sporulation have also been recorded in fungi. Peptide antibiotic accumulation accompanies conidial production in *Pithomyces chartarum* when growth rate falls following nitrogen depletion. The concentration of sporidesmolides seem to be proportional to the number of conidia formed. The onset of sporulation is also associated with change in activity of several enzymes such as phenol oxidase, protease, etc. A correlation between laccase formation and morphogenesis of perithecia in *Podospora anseriana* has been demonstrated. Phenol oxidases are also implicated in the development of phenolic pigments in a number of wood rotting polypores. In *Penicillium griseofulvum*, conidiation was induced by the exhaustion of nitrogen supply and at the same time there was an eight fold increase in the protease activity. Addition of nitrogen source prevented conidial development while a sharp decline in the accompanying protease level was noticed. It is obvious that protein turnover is a basic function of fungal metabolism and an increased proteinase activity is essentially connected with conidiation under some situations as nitrogen depletion.

Fruit Body Formation in Pure Cultures of Basidiomycetes

The formation of fruit bodies in basidiomycetous fungi is the most dramatic expression of morphogenesis and differentiation among fungi. Besides physical stimuli such as reduced temperature or photoinduction, stimulation of fruit body formation under the influence of chemicals as well as microbial metabolites have been reported in literature. **Davis** and **Jong** (*Mycologia* 68:211–14, 1976) reported that *Laccaria laccata* produced normal and mature basidiocarps when cultivated on Rabbit food agar under diurnal alteration of darkness and light after 14 to 16 days at 24 °C. **Ward** et al. (*J. Gen. Microbiol.* 104:23–30, 1978), observed increased production of ethylene during development of sporocarps in *Agaricus bisporus* and that the mycelium grown in compost was responsible for the ethylene production. **Akita** et al. (*Trans. Mycol. Soc. Jpn.* 23:475–79, 1982), produced fruit bodies of the rare basidiomycete *Aseroe arachnoidea* on rice straw or chaff saturated with nutrient media containing glucose, malt extract, ammonium nitrate and mineral salts under sterile conditions. Mycelial growth was best at 30

Lentinus edodes (**Terashita** et al. *Agr. Biol. Chem*. 45:1929–35, 1981). **Azuma** et al. (*Agr. Biol. Chem*. 54:1441–46, 1990), isolated from a soil isolate of *Streptomyces rubiginosus*, a substance designated basidifferquinone, which induced formation of incomplete fruit body or stipes without pileus of *Favolus arcularius*. Stipe formation was induced at 5 ng/ml levels which differentiated to form pileus upon successive photoirradiation.

CHAPTER 5

Strain Improvement

Metabolite Overproduction and Secretion of Heterologous Proteins

The discovery of a new and economically useful metabolite in microbial systems is followed by focused efforts to enhance its level of production through strain improvement and selection. Improved production of the desired metabolite(s) is achievable through innovative approaches in strain selection coupled with careful optimisation of the parameters of the fermentation technology. It is recognised by microbiologists and fermentation technologists that strict regulatory controls exist in wild strains of microorganisms isolated from the natural environment. Such controls govern the synthesis of essential metabolites required for the growth of the organism from the limited quantities of nutrients available from the environment, thereby avoiding wastage and overproduction. To optimise production of the desired metabolite(s), one of the primary requirements would be the deregulation of the rigid metabolic controls through alterations induced in the genetic makeup of the natural isolates by mutation. In the prokaryotic bacterial and Actinomycetes systems, mutagenesis by physical and chemical mutagens followed by selection through innovative approaches have led to several success stories in achieving hyperproduction. For example, mutants blocked in various steps of their metabolic pathway in the biosynthesis of intermediate metabolites (auxotrophic mutants) have been successfully applied to the large scale manufacture of different amino acids, notably lysine and glutamic acid by

Corynebacterium glutamicum. In case of secondary metabolites like antibiotics as well as industrial enzymes, improved production levels are attained through the coordinated action of multiple genes and often the basis for enhanced activity stems from more complex processes of alterations in the genetic regulatory mechanisms. The eukaryotic nuclear organization of the fungi is much more complex than the bacterial genome. Also the filamentous nature and multicellular organisation of the fungi adds a further dimension to be reckoned with, when considering strain improvement and enhanced metabolite production.

Industrially useful fungal cultures are generally those which lack a sexual cycle and are in the haploid phase, reproducing through asexual spores. Heterokaryosis and parasexual cycle are known to take place in these asexual strains and sometimes diploidisation through parasexual cycle also takes place. These are contributory factors leading to uncontrolled and often undesirable variations in the genetic stability of production strains. Conservation techniques without resorting to frequent subcultures of stock cultures and keeping them continuously in the active growing phase, minimise the risk associated with strain degeneration through the operation of these phenomena. It would be particularly necessary for the fermentation mycologist to ensure that such variations do not lead to decline in productivity of strains that have been selected for high yields of the desired metabolite(s) following mutation and innovative screening procedures. It would be obvious that strain improvement programmes must give equal emphasis on the standardisation of procedures to ensure long-term conservation without degeneration during progressive serial transfers. Mutants and variants which are genetically unstable may require to be identified early and eliminated since they would not be very suitable for industrial process development. An understanding of the mycological basis for high productivity would be very helpful in undertaking studies on strain improvement. Well-defined morphological characteristics, if associated with high productivity, can help focus on selecting strains after mutation conforming to the identified morphological features. For

example, in some cases high antibiotic productivity would be associated with strains showing feeble sporulating heterokaryons, which if allowed to sporulate freely, would segregate into homokaryons with associated loss in the high productivity characteristic. In such cases, the stock cultures may necessarily require to be conserved effectively only in the mycelial state and under conditions which do not promote free sporulation of stock cultures.

Strain Improvement

The term strain improvement essentially refers to the identification of variant(s) of the original (parental) organism with an altered genotype which is considered better suited for its application in the fermentation process technology. The selected strain, while being better suited to our objectives, may be a weakened and degenerative one as far as its other biosynthetic capabilities are concerned and consequently in its adaptability and ability for survival in the natural environment. From a natural population of a pure culture, single spore isolates are selected, grown and evaluated for the degree of variation prevailing among the different progenies of the wild strain in regard to their ability to produce the desired metabolite. This procedure of natural selection will enable a preliminary screening and selection for those variants which have the natural capability and genetic makeup for higher productivity compared to the rest of the population. In the next step, mutations can be induced followed by selection of colonies to identify those with higher productivities. Conventionally physical mutagens like ultraviolet and ionising radiations as well as chemical mutagens like NTG (*N*-methyl *N*′-nitro-*N*-nitrosoguanidine) have been employed to treat spores or mycelial fragments. It has been recognised that different effects are produced by different mutagens. Ionising radiations bring about alterations and gross chromosomal changes, while ultraviolet radiation results in small structural changes in the genes through point mutations. Chemical mutagens like phenethyl nitrogen

mustards and ethyleneimino pyrimidines may show considerable increase in mutation frequencies without extensive lethal killing of the spore populations exposed to them. It is customary to use a range of mutagens in succession in the course of selection for mutants with enhanced productivities.

Following mutagenesis, success in the identification of superior mutants is generally a labour-intensive process if only mechanical screening of hundreds of colonies in comparison with the parent strain are to be evaluated in laboratory scale fermentations. A noteworthy example is the increase in penicillin production achieved from 250 units per ml in 1945 to 10–15 × 10

cephalosporin overproduction in the case of *Cephalosporium acremonium*. The complex biochemical pathways associated with antibiotic synthesis make it obvious that their synthesis would be regulated by intrinsic feedback mechanisms. Overproduction can be achieved through selection of mutants in which feedback inhibition or repression is either absent or substantially reduced. **Ball** (*Folia Microbiologia* 25:524-31, 1980) considered metabolic changes that give rise to productivity improvements, and noted that it led to questions about steps limiting product formation as well. It is more than likely that a plurality of steps catalysed by enzymes are involved and alteration of any one or a number of enzymes might nevertheless increase productivity in such a strain. Indirectly such enzymatic changes may affect the properties of the so-called limiting enzyme and also the availability of the substrates. From the foregoing discussion the complexity of the problems facing the microbial geneticist undertaking strain improvement programmes in fungal system is brought into focus. It must also be understood that in mutation and strain improvement programmes, quantum enhancement in yields are attained in early phases of strain selection, but the selection procedure becomes more critical during the later stages of a programme when the improvements are usually small compared with the earlier rounds. The number of innovative approaches applicable to the selection of superior strains after mutation is infinite and depending on the fungal system under study, successful isolation of high productivity strains would be feasible through an intelligent application of a combination of the various techniques available and used successfully in several industrial laboratories.

Under practical conditions of bioproduct manufacture, successful achievement of high titres is the result of an integration between strain productivity and the optimised fermentation environment. Many genes, if altered may be responsible for the overall productivity improvement. Also factors such as the ratio of carbon nutrition utilised for growth and maintenance by the individual strain in the production unit will be significant. Genetic control of mould morphogenesis which, in turn, is influenced by levels of oxygen

uptake and other environmental factors could have a very significant part to play in the expression of high productivity by promising strains under user conditions. It is obvious that strain selection is only one of the constituent steps which needs to be carefully integrated with optimised fermentation parameters such as instrumentation control, dosing and gradient feed addition of specific nutrients, pH control etc. in order to achieve manufacturing success with high yields.

Genetic Transformation

Modern techniques of recombinant DNA facilitate manipulation of the genome of organisms. It enables the correlation of in vitro studies on purified DNA with their biological consequences through transformation in vivo. The yeast *Saccharomyces cerevisiae* and the filamentous fungi *Neurospora crassa* and *Aspergillus nidulans* have been the model organisms in which the techniques for DNA-mediated transformation have been optimised. These techniques have found widespread application and presently transformation studies have been reported in diverse fungal groups ranging from Oomycetes to Basidiomycetes.

Fincham (*Microbiol. Rev*. 53:148-70, 1989) has comprehensively reviewed transformation in fungi which can be consulted for more details, while only basic facts of fungal transformation are discussed here. Transformation is becoming increasingly important in modern mycological research towards a better understanding of fungal genome organisation and for applications in biotechnology. Preparation of fungal protoplasts using standard procedures involving cell wall digesting enzyme mixtures (e.g. Novozym 234) is the first step commonly carried out in transformation studies. Germinating spores or young mycelia, can be used as starting material. Protoplasts which are osmotically stabilised in the presence of salts such as sodium chloride, magnesium sulphate or polyols such as sorbitol and mannitol can be stored at –70 °C for later use. Uptake of DNA (linear or double stranded circular) by

protoplasts is carried out in the presence of calcium ions followed by high concentrations of polyethylene glycol (PEG).

An alternative simple technique for transformation in *Neurospora crassa* which does not involve protoplasts was reported by **Dhawale** et al. (*Current Genetics* 8: 77–79, 1984). Germinated conidia treated with lithium acetate were incubated with DNA and exposed to polyethylene glycol. A brief heat shock treatment followed by plating on a selective medium resulted in the isolation of transformants in high numbers. Circular DNA, linear plasmid DNA and also genomic DNA were successfully used in the transformation studies.

Electroporation of protoplasts in *N. crassa* (**Kothe** & **Free**. *Fungal Genetics Newsletter* 43:31–33, 1996) or germinated conidia of *A. nidulans* (**Sanchez** & **Aguirre**. *Fungal Genetics Newsletter* 43:48–51, 1996) has been successfully applied for fungal transformation. Several dominant selectable markers have been identified for use in transformation. These include auxotrophic markers, use of chlorate resistance generating mutants lacking in nitrate reductase followed by using *A. nidulans* niaD gene as a selectable heterologous marker, selection for selenate resistance, fluoroacetate selection of acetyl coA synthetase mutants etc. Drug resistance markers have been of much usefulness such as for example benlate resistant β-tubulin gene of *N. crassa* or oligomycin resistant gene in *A. nidulans*. Resistance to hygromycin has been employed as the sole selectable marker in study of *Ustilago maydis* mating types (**Komper** et al. *Cell* 81:73–83, 1995).

In recent years successful application of *Agrobacterium tumefaciens* mediated transformation of filamentous fungi have been reported. **De Groot** et al. (*Nature Biotechnol*. 16:839–42, 1998, *Chem. Abst*. 129:299987, 1998), reported that as in the case of plant cells, *A. tumefaciens* could also transfer its T-DNA efficiently to the filamentous fungus *Aspergillus awamori* and the transformation of protoplasts and conidia were improved up to 600-fold in frequency compared with conventional techniques of *A. awamori* protoplasts. The T-DNA has been integrated into the *A.*

awamori genome in a manner similar to that observed in plants. A wide range of fungi were successfully transformed by the *A. tumefaciens* system indicating the applicability of the technique in mycological studies. In a patent application filed by **Unilever**, Netherlands (*Chem. Abst.* 129:287764, 1998) it was observed that transformation using *A. tumefaciens* facilitated one expressible gene or even multiple copies of such genes could be targeted. Also these multiple copies could be of a gene encoding a desired homologous or heterologous protein. Transformation of *A. awamori* using a highly virulent *A. tumefaciens* to deliver a binary vector designed for use in fungi gave 300–7200 transformants per 10

heterologous proteins and manufacture mammalian proteins such as chymosin in fungal systems. A brief discussion on some of the approaches involving innovative strain selection as well as molecular techniques as applied to fungal enzyme hypersecretion is presented here along with a discussion on the current trends in heterologous protein secretion in fungal systems.

Intensive research to isolate strains of fungi such as *Trichoderma reesei* for hyperproduction of cellulase have led to selection of mutants characterised by high activity and secretion of extracellular protein in excess of 30 grams per litre under appropriate and optimised fermentation conditions. From the wild strain designated QM 6a, mutant strains QM 9123 and QM 9414 were identified at the U.S. Army Natick Laboratories, while a semi constitutive mutant RUT-C 30 was identified at the Rutgers University, USA. Selection for catabolite repression resistance is achieved by looking for colonies exhibiting cellulose clearance in presence of glucose or colonies which are resistant to inhibition by glucose analog, 2-deoxy glucose. **Durand** et al. (*Enz. Microbial Technol.* 10:341–46, 1988) have discussed genetic improvement of *T. reesei* for large scale cellulase production and have described the selection of a hyperproducing mutant designated as CL847 from QM 9414 by a series of mutagenic treatments and this improved strain produced four-fold increased cellulase activity in cellulose media but also produced the enzyme in simple media containing the soluble sugar, lactose. Practical aspects for industrial strain selection emphasised include strain stability, good sporulation and lack of fastidious nutritional requirements. Also designed were screening procedures for cellulase hyper-producers which are derepressed mutants as those which are constitutive for β-D-glucosidase as well as for endo and exo-1, 4 β-D-glucanase.

Alkaline protease production by *Fusarium* sp. growing on *n*-paraffin could be significantly enhanced by mutagenesis and selection for resistance to an antifungal polyene antibiotic, Kabicidin. The parent strain could not grow at Kabicidin concentration of 20 μg/ml whereas active growth of the mutant strain was observed in a medium containing 50 μg/ml of the

antibiotic. The polyene antibiotic inhibits the functioning of the cell membrane of the fungi. The mutant strain produced ten-fold enhanced alkaline protease compared with the parent strain (**Nakao** et al. *Agr. Biol. Chem*. 37:1223–24, 1973), **Suzuki** et al. *Agr. Biol. Chem*. 38:135–39, 1974).

Molecular studies related to industrial enzyme production from fungal strains have been primarily concerned with a better understanding of the regulation of transcription and to produce strains with different enzyme profiles through genetic manipulation. Regulation of gene expression is a critical factor to be considered. Secretion process and aspects related to the molecular events involved in protein secretion may also be important factors governing the yield of targeted proteins. In amyloglucosidase production, introduction of multiple gene copies has been tried. The complexity of DNA-protein interaction in the expression of the glaA gene encoding glucoamylase has been shown to be dictated by the availability of transcription factors at high gene copy numbers.

Strain stability and consistency of performance in large volume fermentations are major factors to be considered in the application of genetically engineered strains during commercial manufacture. A thorough understanding and critical monitoring of the fermentation parameters will be essential for achieving commercial success with recombinant fungal strains. Recent advances in molecular techniques include development of methods for the manufacture of protein using fungal expression hosts carrying genetic expression cassettes integrated into the host genome. This method is claimed to allow rescue of plasmids and their flanking sequences from transformants showing high level expression of the gene. The use of this method to develop *Aspergillus oryzae* hosts expressing lipase gene of *Humicola lanuginosa* has been described in a patent granted to **Novo Nordisk** (US Patent 5,955,727, *Chem. Abst.* 131:256405p, 1999). Some insertions gave rise to morphological mutants that were useful in fermentation because they were easier to aerate than the wild type and gave higher yields.

Heterologous Protein Secretion by Fungi

Filamentous fungi by virtue of their being considered as safe and high level of protein secretion have received considerable attention as potential hosts for the expression and secretion of heterologous proteins. The general strategy has been to clone a highly expressive fungal gene, for example alpha amylase or glucoamylase and use its promoter for the expression of the heterologous gene. In many cases, regrettably, the yields of the secreted heterologous protein has been very low. Exceptionally, yields in excess of three grams per litre of the naturally secreted *Rhizomucor miehei* aspartic protease were obtained in *Aspergillus oryzae* using an alpha amylase promoter (**Christensen** et al. *Biotechnology* 6:1419–22, 1988).

Bovine chymosin has been an enzyme of intensive study for heterologous production in fungal hosts. The promoter of glucoamylase (glaA) and secretion signal have been used to direct chymosin production in *Aspergillus nidulans* and *A. niger* var. *awamori*. Several expression vectors were made incorporating prochymosin cDNA with either the glucoamylase or chymosin secretion signal. Increased production of secreted chymosin was obtained by fusing the chymosin cDNA to the last codon of the *A. niger* var. *awamori* glaA gene. Mutants were isolated with higher chymosin secretion which were associated with deficiency of aspartyl protease. **Dunn-Coleman** et al. (*Biotechnology* 9:976–82, 1991), have reviewed the commercial levels of chymosin production by *Aspergillus*. A recent review by **Punt** et al. (*Trends Biotechnol*. 20:200–206, 2002), has comprehensively discussed the use of filamentous fungi as "cell factories" for heterologous protein production. Some of the key points discussed are as follows:

Heterologous protein secretion levels are low compared to proteins of fungal origin. Abundant production of secreted proteases cause low yields. The development of improved endogenous protease-deficient host strains largely alleviated the problem of extracellular degradation. Based on the idea that secreted fungal proteins can act as a carrier for more

efficient secretion of a heterologous protein, gene fusion technique was used. Improved production of chymosin was reported in *Aspergillus niger* by expression as a glucoamylase–chymosin fusion (**Ward** et al. *Biotechnology* 8:635–40, 1990). Many examples illustrate this fusion system, although much of the background remains unclear. In some cases, however, fusion fails to improve the yields of secreted proteins. The gene fusion technique still remains the first choice in attempts to produce nonfungal proteins in *Aspergillus*.

While the past decade has provided an abundance of new data regarding the use of filamentous fungi as "cell factories" for heterologous protein production, the basis for strain improvement strategies lies in the development of molecular tools. These tools have initiated a detailed analysis of fungal secretion pathway. It can be concluded that with further advances in our knowledge molecular techniques would play more significant roles in the selection of strains through a combination with classical techniques of mutation and strain selection. The following additional references are also recommended for getting a deeper insight into this topic:

1. **Archer and Peberdy**. The molecular biology of secreted enzyme production by fungi, *Critical Rev. Biotechnol.* 17:273–306, 1997.
2. **Archer**. Biotechnology of filamentous fungi: Applications of molecular biology. In *Molecular Fungal Biology*, edited by **Richard Oliver** and **Michael Schweizer**). Cambridge University Press, 1999: 341–364.

CHAPTER 6

Morphogenesis of Mould Growth in Submerged Culture

Submerged cultivation to produce mycelial biomass of filamentous fungi can be viewed from the point of two different objectives: (a) Production of maximal mycelial growth through optimal and complete utilisation of nutrients to obtain protein-rich Mycelial Biomass Product (MBP) for use in animal and human nutrition. The fungal strain could be a nontoxic nutritionally accepted hyphomycetous species such as *Penicillium chrysogenum* or *Fusarium graminearum* on which a commercial product has been marketed for protein supplement in human nutrition. Cultivation of mycelial biomass with the desired flavour of higher fungi such as *Agaricus bisporus* or *Morchella esculenta* would also belong to this category; (b) Submerged cultivation of moulds to produce various bioactive metabolites such as antibiotics and for this the strategy would involve restricting the amount of nutrients utilised for biomass growth and diverting the major quantity towards the synthesis of the secondary metabolites. Obviously the amount of mycelial biomass generated would be comparatively more restricted.

Cultivation of filamentous fungi in submerged culture has some unique problems, which are not faced while growing either bacteria or unicellular yeast cultures. Inoculum build-up and cell level standardisation of inocula through optical density measurements of cell suspensions are relatively simple with bacterial and yeast cultures. With

filamentous fungi, heavy spore suspensions need to be prepared and suspensions of spores from agar slopes, even in the case of heavily sporulating species such as *Aspergillus* and *Penicillium* fail to provide adequate spore counts. In addition, in slant cultures, physiological heterogeneity in the age and maturity of different spore heads could also be a factor in few cases. Mass sporulation methods for sporulation of commercially employed filamentous fungi have been described. For example, the fungus is inoculated into pearl barley (30 grams in 500 ml Erlenmeyer flasks) and autoclaved at 15 lbs pressure for two hours. Spore suspension is prepared from an agar slant culture in a sterile solution containing 3% (w/v) glycerol and 0.1% L-asparagine and 10 ml of this suspension is used to inoculate one flask of pearl barley. The flasks are shaken thoroughly for uniform mixing of the inoculum and incubated at optimum temperature (usually 25–30 °C). The fungal growth on the pearl barley grains rapidly yields dense masses of spores in a period of 7–10 days from which spore suspensions of desired spore concentrations can be readily prepared for initiating the fermentation experiments (**Whiffen** & **Savage**. *J. Bact*. 53:231–40, 1947). **Thirumalachar** and **Gopalkrishnan** (*Hindustan Antibiotics Bull*. 3:69–70, 1963) outlined a protocol using pearl barley, honey and peptone for mass sporulation of *Penicillium chrysogenum* as well as other filamentous fungi and Actinomycetes. According to this procedure, one hundred grams of pearl barley (*Hordeum vulgare*) of good quality are weighed into clean dry Erlenmeyer flasks and sterilised dry in an autoclave for 90 minutes at 15 lbs pressure. These are taken out and allowed to cool at room temperature. In separate tubes 20 ml sterile solution of 6% honey and 1% peptone in water is prepared. 5 ml spore suspension from an agar slant, soil master culture, a lyophil or another sporulating barley flask is taken and mixed with 20 ml of honey peptone solution. The mixed suspension is then poured into the barley flask and shaken vigorously to disperse the spores evenly and get a uniform coating over the surface of the barley grains. The volume of the solution is usually just sufficient to wet the surface of all the grains. Too much of the solution tends to clump the grains and encourage mycelial formation and too

little tends to reduce the quantum of sporing. Addition of 0.5% sodium chloride to the honey peptone solution encourages the sporulation. Glucose or glycerine–asparagine solution can be used with equally good results for *Streptomyces*. For *S. aureofaciens* and *S. rimosus* starch containing media were very suitable. Periodic shaking to break the clumps and separate individual barley grains is very essential to obtain good sporulation. The authors have reported that several species of *Fusarium, Curvularia, Alternaria, Metarrhizium, Pyricularia, Helminthosporium* and *Paecilomyces* have been successfully cultivated and large quantities of spores obtained. Each flask gives 20 to 30 billion or more spores. Microscopic examination reveals that the mycelium first forms a thin felt round the barley grains. Following sporulation the mycelium breaks down releasing the powdery spores. The remnants of the ephemeral mycelium remain on the barley grains when the spores are removed by washing with sterile water. The method has the advantage of giving uniform quantity of spores for production as well as research studies. By this method it is also possible to make the sparsely sporulating mycelial types of high yielding industrial strains to produce sufficient inoculum for production purposes.

When spores are used as inoculum, a 12–18 hours lag period is observed before significant mycelial growth is observed. The pattern of growth as well as morphogenesis of this growing mycelium is of vital importance in the manufacture of bioproducts. Mycelial morphology strongly influences mycelial growth and the factors underlying the morphogenesis have been the subject of intensive studies in industrially important fungi like *Penicillium chrysogenum* and *Aspergillus niger*. The form of mycelial growth during fermentation affects production kinetics and also determines the rheology of the broth, by causing diffusion limits on nutrients within the mycelial flocs. The mycelial growth can be filamentous, pelleted or any combination of these two extremes in a fermenter. Morphogenesis of mould growth can be studied in shake flasks as well as in stirred tank reactors with forced aeration and agitation. There are significant differences in the parameters influencing growth and morphogenesis in the two

systems. In shake flasks, the submerged mycelial growth is subjected to the amplitude of shaking depending on the speed of rotation of the shaking machine and the aeration needs are met by the column of resident air above the medium in the flask as well as through air diffusing through the cotton–wool plugs. In the stirred tank reactor on the other hand, the sterile air is passed through a sparger and is dispersed into fine bubbles through high speed agitation system of the impeller blads. The mechanical disruption and the shear force of the stirrer system have significant impact on the growing mould cultures and their morphogenesis.

Types of Mycelial Growth

Fungal growth in submerged culture takes place in three forms and they are often found to be overlapping, without any sharp differentiation between them. The three forms recognised are single cell, filamentous and pelleted growth forms. In single-cell growth form, spherical to ellipsoid cells result from the high shear conditions prevailing in the fermenter. In some fungal strains, formation of sprout cells arising out of growing mycelia can also contribute to populations of single cells under submerged culture conditions. Pellet formation in submerged culture has been widely investigated from a mycological point of view. It is generally recognised that the formation of pellets is greatly influenced by the type of inoculum, composition of the culture medium and cultural conditions (**Galbraith** & **Smith**. *Trans. Brit. Mycol. Soc*. 52:237–46, 1969). Several mechanisms can be distinguished for pellet formation, viz. agglomeration of spores, agglomeration of solid particles and hyphae, pellet–pellet and pellet–wall (fermenter wall, baffles, cooling coil, stirrer blades etc.) interactions and these are considered important for pellet formation. **Metz** & **Kossen** (*Biotechnol. Bioeng*. 19:781–99, 1977) surveyed and presented a literature review on the growth of moulds in the form of pellets. These authors distinguished three types of pellets: (a) fluffy loose pellets (hairy pellets with a compact centre and a much looser

outer zone) (b) compact smooth pellets (the whole pellet is compact and the outside of the pellet is smooth) and (c) hollow pellets (the centre of the pellet is hollow owing to autolysis and the outside of the pellet is smooth). **Pirt** and **Callow** (*Nature* 184:307–10, 1959) observed a link between pellet formation and the formation of short distorted hyphae. The most important advantage of growth in the pellet form is attributed to a considerable decrease in the broth viscosity, in comparison to growth in the filamentous form (**Metz** et al. *Advances in Biochemical Engineering* 11:103–56, 1979). **Steel** et al. (*Can. J. Microbiol*. 1:150–57, 1954) found formation of pellet type cultures was desirable for citric acid fermentation with *Aspergillus niger*. However, cultivation in the pellet form may, in some cases, introduce undesirable limitations and the most important of these is the diffusional limitations of essential nutrients like carbohydrates and also oxygen into the deeper layers of the mycelial pellets. When the radius of the pellet exceeds a critical value, only a peripheral zone of the pellet will contribute to growth. **Philips** (*Biotechnol. Bioeng*. 8:456–60, 1966), observed that low penicillin productivity occurred when large pellets of *Penicillium chrysogenum* were formed and very high dissolved oxygen levels were necessary to obtain reasonable productivity when the mould growth was pellety. Therefore it appeared that insufficient oxygen diffusion into mycelial pellets might be the factor that limited the productivity under the prevailing conditions. The oxygen utilisation rate and apparent critical oxygen tension of mycelial pellets were predicted on the basis of diffusion theories and the results indicated that oxygen supply to the interior of the pellets was through molecular diffusion. With large pellets it was relatively easy to maintain high dissolved oxygen levels in fermentation broths, but it was difficult to ensure adequate oxygen supply to the interior of the pellets. Conversely, the smaller the pellets, the more difficult it was to maintain high dissolved oxygen levels in the broth but the oxygen could be more readily transferred to the interior of the pellets. **Van Suijdam** and **Metz** (*Biotechnol. Bioeng*. 23:111–48, 1981) analysed the influence of engineering variables on the morphology of filamentous moulds in submerged culture. A dynamic equilibrium existed between

growth and break-up of hyphae depending on the shear stresses in the fermenter. Studies on *Penicillium chrysogenum* in batch as well as continuous culture revealed that the length of the mycelial particles increased with increasing growth rates and decreased with increasing power inputs per unit mass in the fermenter. It was observed that oxygen tension varied independent of stirrer speed and had little influence on mould morphology. Possible use of high energy inputs in industrial mould fermentations in order to decrease hyphal length was regarded as having little commercial potential since an enormous increase in energy inputs would have to be entertained.

Various factors which influence growth conditions also influence formation and structure of mycelial pellets. Some of the factors are listed below.

Agitation

A primary effect of agitation relates to the dispersion of spore aggregates and agglomerates in the medium. The number of spores per agglomerate was inversely proportional to the power input in the fermenter. Thus strong agitation can prevent formation of pellets for fungal strains with a tendency towards coagulative pellet formation. In moulds like *Mucor* and *Rhizopus* characterised by non-septate mycelia, pellet formation only occurred under vigorous agitation conditions. Pellet formation in Basidiomycetes like *Agaricus* or the edible Ascomycete *Morchella* was favoured under conditions of low aeration and low speed agitation and filamentous growth resulted with increased aeration rates.

Growth Medium

In *Myrothecium verrucaria*, high nitrogen concentrations (100–500 nmol of ammonium nitrate) gave compact and smooth pellets, whereas loose and fluffy pellet formation took place with low nitrogen levels (1–5 nmol) (**Darby** & **Mandels**. *Mycologia* 46:276–88, 1954). Ammonium sulphate in the medium promoted pellet growth while cornsteep liquor

addition as the source of nitrogen resulted in a pulp-like growth in *Penicillium chrysogenum* (**Pirt** & **Callow**. *Nature* 184:307–10, 1959). In *Aspergillus niger*, source of nitrogen as well as the amount of phosphate in the medium strongly influenced the formation of fluffy loose pellets during citric acid formation. Trace metals like manganese, iron and zinc are also significant in determining morphogenesis. When grown on synthetic media, addition of chelating agents such as EDTA as well as ferrocyanide resulted in the formation of smaller and more compact pellets. (**Choudhary** & **Pirt.** *J. Gen. Microbiol*. 41:99–107, 1965). Fatty acid addition strongly influenced pellet growth in the mucoralean fungus, *Mortierella vinacea*. Addition of 0.1–0.3% oleic acid caused increase in growth rate, enhanced enzyme activity and a decrease in pellet density (**Kobayashi** & **Suzuki**. *J. Ferm. Technol*. 50:625–29, 1972).

pH

Pellet formation and especially the coagulation of spores is strongly influenced by the pH of the growth medium. In *A. niger*, coagulation was most pronounced at pH 5.0 while no coagulation took place at pH 2.0. This suggests that surface properties of the spores that are influenced by pH are responsible for their coagulation behaviour.

Oxygen Tension

Oxygen diffuses into the pellets and is consumed by the growing fungal mycelium. Hence oxygen tension in the medium is very important for ensuring sufficient oxygen supply to the pellets. Due to the limited solubility of oxygen, rapid depletion of oxygen in the centre of the pellets may readily take place. As a result, autolysis of the cells and eventual formation of a hollow centre can be observed. In *A. niger*, aeration with air produced fluffy loose pellets which became hollow, when the pellets were larger than 1.75 mm in diameter (**Clark**. *Can. J. Microbiol*. 8:133–36, 1962). Aeration with gas containing 50% oxygen produced a much denser

growth at the outer region of the pellet in which the hyphae appeared shorter, thicker and branched to a greater extent. These compact pellets showed autolysis at diameters greater than 1.0 mm illustrating the influence of pellet density on the diffusion of oxygen into the interior zones.

Inoculum

It is generally recognised that the concentration of inoculum significantly influences pellet formation. A high concentration of spore inoculum is regarded as conducive to filamentous mycelial growth while low spore concentrations produced pellets. Hyphal interactions influence and determine the possibilities of pellet formation. At high concentrations, the hyphae interact with one another in the early stages of growth, thereby preventing the development of pellets. This is supported by the observation of **Pirt** and **Callow** that pellet formation in a strain of *Penicillium chrysogenum* was more pronounced when the hyphae were short.

Polymer Addition

Takahashi et al. (*J. Agr. Chem. Soc. Japan* 84:440, 1960), observed a shift from pellets to pulp-like growth of *A.niger* when they increased the viscosity of the medium from 1 to 40 cP in shake flasks by the addition of sodium alginate and dextran. In contrast, **Kobayashi** and **Suzuki** (*J. Ferm. Technol*. 50:835, 1972) found that there was an increase in pellet size in *Mortierella vinacea* when in the medium viscosity shake flasks was increased to 80 cP by addition of sodium carboxylmethyl cellulose. The main effect of polymer addition is the change in the morphology of the mould. Effect of spore agglutination caused by polymer addition prior to germination seems to be most significant. Examples of polymers added include carboxypolymethylene (carbopol 934) or Retan, a polyacrylate which produced in their presence a much dispersed growth. **Trinci** (*Trans. Brit. Mycol. Soc*. 83:408–12, 1983) studied the effect of addition of water soluble acrylic resins, Junlon 110 and 111 on the morphology of *A. niger* in

shake flask cultures. At 2 grams per litre level, the growth was transformed from the conventional pellet form into finely dispersed filamentous mycelia which could be reliably evaluated by culture turbidity for the measurement of the amount of biomass formed.

While compared with the pellet form of growth, the filamentous growth is composed of tangled hyphal masses leading to a suspension, that can be very viscous. The rheological behaviour is usually very non-Newtonian with relatively low viscosities in regions of high shear rate near the impeller and very high viscosities in regions with low shear rate near the fermenter wall. High viscosity conditions lead to problems in the mass transfer from the gas to liquid phase and considerable depletion of oxygen to the growing mycelia. Besides, problems in uniform mixing as well as heat transfer are also encountered.

In a recent review, **Gibbs** et al. (*Critical Rev. in Biotechnol.* 20:17–48, 2000) have analysed the problems related to growth of filamentous fungi in submerged culture, with particular reference to the influence of physical and chemical environment on culture morphology, process engineering challenges posed by different fungal morphologies and the relationship between fungal morphology and metabolite production. For more details and critical information than what has been presented, this review may be consulted. Correlation between morphogenesis of growth and product formation would be very desirable not only from the point of academic interest but also from the point of enhancing productivity. An interesting correlation between morphogenesis and extracellular alkaline protease secretion in *Basidiobolous haptosporus* has been reported from our laboratory (**Ingale** et al. *World J. Microbiol Biotechnol*. 18:403–408, 2002). The manner in which the inoculum was developed ('Darmform' cells or mycelial inoculum) determined the growth morphogenesis in the experimental flasks as dispersed fine pellet growth or as very large pellets. High levels of alkaline protease secretion was only observed when free cell type of 'darmform' inoculum producing finely dispersed pellet growth took place while large pellets growing from mycelial inocula showed negligible protease secretion.

Conclusion

Morphogenesis in submerged culture and the underlying parameters governing it are presently understood based on the limited studies carried out on select heavily sporulating industrially useful moulds, notably *Aspergillus* and *Penicillium*. As a wider range of fungal systems become important in industrial biotechnology, their morphogenetic behaviour in submerged culture would require to be given increased attention and may be expected to throw newer challenges in their study and understanding. Several bioengineering problems which are associated with morphogenesis of the culture growth have been recognised and innovative solutions worked out. Often in relation to individual strains, closer interaction between mycologists and process engineers would be desirable even from the early stages of technology development since it will enable getting a better grasp of the scale up problems and for which innovative solutions could be worked out through the collective efforts of the biologists and the engineers.

CHAPTER 7

Solid State Fermentation

Solid State Fermentation refers to the utilisation of water insoluble substrates for microbial growth and in which fermentation is carried out under conditions where availability of free liquid is highly minimised. This is in contrast to the submerged culture system, where the nutrients are well dispersed in a liquid medium and the fermentation parameters optimised for submerged growth with uniform mixing of the culture with the medium through shaking or stirring during the growth phase. Solid substrate fermentation is also distinct from surface culturing on liquid media or media containing low concentration solid slurries. At moisture contents less than 12%, growth generally does not take place and thus Solid State Fermentation (SSF) could be manipulated to varying levels above this minimum level and fermentations carried out. Some of the traditional examples of SSF practised for a long time include composting, cheese making and also the fermented foods of the orient. The "koji" process for microbial fermentation on solid substrates like cereal brans has been widespread in countries like Japan. This process has been successfully implemented for the manufacture of a wide range of microbial metabolites. Solid substrate fermentation of starchy foods to enhance their protein content and nutritional value has also sustained considerable research interest. Upgrading the protein content through microbial fermentation of cellulosic feeds for animal

nutrition has also received much attention and process development by SSF attempted.

SSF technology is generally recognised as being simpler and requiring less energy for processing compared with submerged processes. The amount of liquid per unit mass of substrate is low and the requirements for aeration and mixing are comparatively simpler. However SSF processes have the disadvantage of being relatively slower due to the barriers to penetration and growth of the microbe posed by the bulk density of the substrate. Heat dissipation limitations are also a problem to reckon with when rapid metabolism of the substrate by the growing culture may result in pockets in the SSF with uncontrolled and undesirable rise in temperature.

Filamentous fungi are better suited for growth on solid substrates with low moisture content and successful process technologies by SSF have focussed on (a) mucoralean fungi especially *Rhizopus* and *Mucor* (b) rapidly growing hyphomycetous fungi such as *Aspergillus, Penicillium, Fusarium,* etc. (c) basidiomycetous fungi, especially edible mushrooms like *Agaricus, Pleurotus* and *Volvariella* which are cultivated on composted or noncomposted agro-residues to produce the edible mushroom fructifications.

Solid Substrate Fermentation with Pure Cultures

Under natural conditions a succession of microflora are involved in the breakdown and recycling of organic matter. Several bacteria, actinomycetes and fungi including thermophilic forms are involved in mixed culture fermentations in well-known processes like ensiling and composting. These natural fermentation processes taking place under non-sterile conditions are the result of an orderly succession of microbes colonising and degrading the substrate and effectively recycling the organic matter through their specific chain of enzyme systems. From a practical point of view the substrates suitable for SSF are those which are rich

in starch (grains, tubers, roots, etc.) and/or cellulose/ hemicellulose-rich residues (cereal brans, straw, agricultural and forest residues). Wheat bran is by far the most favoured substrate as it offers several advantages. It is nutritionally well balanced and available in quantity from milling locations and is fairly uniform in composition. *Mucor, Rhizopus* and *Aspergillus* species form good biomass growth on starchy substrates while species of *Trichoderma*, *Chaetomium* or *Penicillium* can break down the cellulose and hemicellulose components of plant biomass to yield mycelial biomass. The end products of SSF fermentations range from protein-rich mycelial biomass to extracellular enzymes and secondary metabolites. Under optimum conditions, levels of biomass and extracellular enzymes in SSF could be much higher than that obtained in submerged fermentation. For instance a 10-fold higher amylase activity was reported within three days in SSF fermentation of rice with *Aspergillus oryzae* (**Bajracharya** & **Mudgett**. *Biotechnol. Bioeng*. 22:2219–35, 1980). In a solid state system, 20–24% crude protein was obtained in five days when alkali-treated corn stover was fermented with *Chaetomium cellulolyticum* (**Chahal** & **Moo-Young**. *Dev. Indust. Microbiol*. 22:143–58, 1981).

Fermentation process technology development by SSF for product manufacture requires cultivation of chosen single strains under strictly controlled conditions of fermentation. In the traditional "koji" process of the orient, the art of fermenting cereal grains and soybean by moulds was standardised for the manufacture of products such as soysauce, Tempeh, Tofu etc. (**Hesseltine**. *Biotechnol. Bioeng*. 14:517–32, 1972). The moulds produced the amylolytic and proteolytic enzymes which degrade the nutrient substrates being fermented. Commercial "koji" process has been adopted for the manufacture of amylase used in the starch-based fermentation for alcohol production. Basically the process involves cooking wheat bran moistened with dilute hydrochloric acid at 100 °C for thirty minutes. The mash at 50% moisture and pH of 3.5 is inoculated with a heavy spore suspension of the amylase secreting *Aspergillus oryzae* strains and spread in trays. After 36 hours of growth at 30–32 °C, the

fungal biomass is harvested and the enzyme extracted from the wheat bran koji. Adaptation of the "koji" process with minor modifications has been published or patented for the production of other industrial enzymes like protease, pectinase and cellulase as well as other secondary metabolites such as antibiotics and mycotoxins. Some specific examples of product manufacture by SSF are described later.

Mixed mycoflora fermentation in SSF has also been attempted and solid state fermentation of wheat straw with greater efficiency of conversion to protein-rich fungal biomass product was achieved with a mixed culture of *Chaetomium cellulolyticum* and *Candida lipolytica* or using *Trichoderma lignorum* in combination with *C. lipolytica.*

Mycological Aspects of Growth in SSF

Unlike the growth pattern in submerged culture, where the fungal hyphae fragment under the impact of the impeller or if the growth is pellety, apical growth occurs at its outer edge, in SSF the growth is principally governed by the capacity of the mycelia to adhere to the solid substrate and under conditions of limited moisture supply, their ability to secrete the metabolic enzymes to solubilise and assimilate the nutrients for growth processes. Structures like rhizoids, haustoria or appressoria present in several fungi aid in their initial anchorage to the particles of substrate during SSF. Actual growth by penetration and ramification of the substrate through secretion of enzymes for its breakdown is strongly affected by several factors including intra-particle mass transfer of oxygen and nutrients from the substrate. Due to the finely interwoven growth of the mycelia with the substrate, it is difficult under SSF conditions to accurately assess the biomass growth by weight determination. Indirect estimates based on the protein content, taking into account corrections for certain interfering values have been standardised. In the case of actively growing mycelial biomass, extent of growth can also be assessed on the basis of ATP

measurement, oxygen uptake, carbon dioxide evolution or glucosamine content. The percentage of cells in the active metabolic state and those in the declining phase of growth and differentiation varies at different stages and contribute to physiological heterogeneity and this must also be taken into account while interpreting the biomass data.

The rates of biomass growth in SSF are much lower than under liquid fermentations. While the average mass doubling time for most fungi is 4–8 hours in submerged culture, estimates under SSF indicate periods as long as 5–6 days. Under submerged cultivation conditions, the parameters for fermentation are much better controlled than under SSF. Contamination can sometimes be serious and variations in fermentations parameters such as gradient feed addition of specific nutrients or pH profiling are not easily practised under SSF systems. In SSF, the parameters on which control can be exercised are the substrate and its surface to volume ratio, pH, temperature and moisture content and the composition of the aeration gas (air, air–CO

Microbiology Washington D.C. 1986, pp. 66–83) reviewing solid state fermentation has discussed process variables as well as process control. It is necessary to appreciate the unique nature of each microbe-substrate system governed by chemical composition and physical properties of the substrate, growth characteristics and physiology of the organism and the nature of the product. Important among the key variables deserving consideration in process development may be mentioned substrate pretreatment, addition of nutrient supplements, particle size, moisture content, inoculum density apart from temperature, pH, aeration-agitation which may also be significant factors. Pretreatment to modify the substrate to render it more accessible to mycelial penetration would be favourable for the fermentation. Alkali cooking of cellulosic residues to break the hydrogen bonds and creating an amorphous cellulosic substrate which is more readily degraded by the fungi and their extracellular enzymes is a good example. As an example of nutrient supplement, mention may be made of adding low levels of glucose to stimulate lignocellulosic degradation by white rot Basidiomycetes like *Lentinus edodes* and *Phanerochaete chrysosporium*. Smaller particle size of the substrate provides larger surface area facilitating better heat transfer and gas exchange. This leads to an overall improvement in surface nutrient concentrations. However it has been also realised that there is a critical minimum particle size below which too much packing density and reduction in void space between particles can occur. This may have an unfavourable impact on heat exchange as well as diffusion of gases. Reduction in particle size to the desired extent is achieved by mechanical means like grinding or ball milling. Moisture content is a critical factor which seriously affects the mould growth as well as metabolite production. Above 40% moisture levels, bacterial contamination in SSF becomes critical. Depending on the substrate and the microbe involved, the optimal moisture content for the fermentation has to be standardised so that bacterial growth is limited and the fermentation proceeds satisfactorily. By such manipulations of the moisture level, successful fermentations can be carried out on fermentation mashes subjected to

steaming at 100 °C instead of regular sterilisation under pressure.

Inoculum density optimisation is also important since very low levels may lead to insufficient biomass build-up, while high levels of inocula may give rise to excessive mycelial growth and relatively less nutrient availability for the production of the desired metabolite. The variation as well as gradients in pH and temperature significantly influence the progress of the solid state fermentations and unlike in the case of submerged fermentations are difficult to precisely exercise controls. Aeration and agitation are also important in SSF. However many of the fermentations are sensitive to manual mixing due to the disruption of mycelial contact with the substrate and consequent interruption of the action of the cell-bound enzymes and their role in solubilising the insoluble substrate matrix to provide essential nutrients for the growing culture.

Increasing interest in the application of SSF for bioproduct manufacture has led to innovative efforts to improve the equipment used and overcome the well recognised shortcomings in the presently employed SSF technology. One such development is the design of a SSF bioreactor designated "Plafractor" by **Biocon (India) Ltd**., Bangalore, India for which international patents have been granted (US Patent No. 6,197,573 dated March 6, 2001). The invention relates to an improved SSF device that combines all the operations of microorganism cultivation (sterilisation, inoculation, cultivation, extraction and post-extraction treatment). The SSF device is modular and operates in a contained manner facilitating an aseptic environment within the bioreactor. Special provisions for heat removal as well as addition of fluids to the bioreactor during fermentation are incorporated in the reactor design.

Metabolite Production under SSF

The following examples give a comprehensive but not exhaustive account of the application of SSF technology for the manufacture of different fungal metabolites. An international symposium on solid state fermentation was held and the proceedings have been published (**Roussos**, **Sevastanios** Ed. *Advances in Solid State Fermentation*, Kluwer Dordrecht, the Netherlands, 1997) and many of the examples cited here are from papers presented at the symposium. **Kabbaj** et al. (p. 437–48) (*Chemical Abstracts* 128:179417) reporting on the factors affecting physiology of mycelial growth and aroma production in SSF using *Pleurotus* and *Morchella* found that a C/N ratio of 10 and sodium nitrate as nitrogen source favoured aroma formation. As the aroma compounds produced by mycelial cells of *M. esculenta* are identical to those formed by the fruiting bodies, a strategy for simple and economic way to produce the highly priced morel mushroom aroma by SSF has been envisaged by the results obtained. **Perraud-Gaime** & **Roussos** (pp. 209–221) (*Chem. Abst.* 128:153372) studied fungal decaffination of coffee pulp to eliminate the antiphysiological effects on animals if used as animal feed and found a strain of *Penicillium* to be most effective for caffeine degradation by SSF prior to the stage of conidiospore formation. **Roussos** et al. (pp. 483–500) (*Chem. Abst.* 128:179393) described a new culture technique for studying the physiology of *Pleurotus opuntiae* mycelium in SSF utilising sugarcane bagasse as solid support for absorbing a liquid culture medium. The method was considered to offer better control of liquid medium composition as well as culture conditions. Under optimum conditions nearly 90% of substrate was consumed in six days, paving way for practical technology used in the development of mycelium inocula for commercial exploitation of *Pleurotus*. **Barrios-Gonzalez** et al (pp. 407–415) (*Chem. Abst.* 128:179388) studied penicillin production in SSF with particular reference to environmental and genetic factors. The results indicated the importance of using high moisture content and concentrated media to obtain high penicillin production. Production is controlled by the

proportion of the support nutrients and water. Highest titres were obtained when the solid medium had low bagasse content (10.3–12.5%). Antibiotic production in SSF is also regulated by catabolite repression, ammonium concentration and feedback regulation. Special strains suited to produce penicillin efficiently under SSF conditions were selected for satisfactory performance. *Rhizopus delemar was grown by absorbing suitable nutrient medium for lipase production into a polymetric resin [Amberlite IRA 900] (***Christian** et al. *Chem. Abst.* 128: 321–24, 179401). In SSF the enzyme activity was 93 U/g dry amberlite at 24 hours in comparison with 14.1 u/ml at 48 hours in submerged culture. Enzyme production could be observed even in the presence of glucose in SSF while it was strongly inhibited under submerged culture in presence of sugars. **Alcaraz-sandoval** et al. (pp. 311–19) (*Chem. Abst.* 128:179400) studied several fungi in SSF for mannanase production as mannan degrading enzymes are useful in the processing of instant coffee, fruit juice clarification etc. Highest enzyme activity was obtained with *Aspergillus ochraceus* grown on copra meal in SSF.

Several other research papers published in a wide range of journals add further proof about the increased interest in the study of fungi in SSF for metabolite production. **Pintado** et al. (*Enz. Microbial. Tech*. 23: 149–56, 1998) investigated citric acid production from mussel processing effluents by *Aspergillus niger* in solid state culture using polyurethane foam particles soaked with the culture medium. The study illustrated that strains with low requirements for nitrogen and phosphorus performed better under SSF. **Tunga** et al. (*Bioprocess Eng*. 19:187–90, 1998) found maximal protease production in SSF by *Rhizopus oryzae* in a spore inoculum containing 2×10

Conclusion

From the foregoing brief account, it will be clear that there has been renewed interest in adopting the classical methodologies of growing mould cultures in the solid state on substrates which are insoluble and with innovative adaptations and modifications to suit individual requirements. Future years will certainly witness further advances in the technological aspects and a greater depth in understanding the physiology and biochemistry of mould vegetative growth and sporulation in relation to metabolite elaboration. The relative advantages as well as the shortcomings would be better analysed in comparison with the submerged fermentation technique and the relative success of the SSF technology would be decided upon by the quantum of yield, ease of processing and the economics of the process.

CHAPTER 8

Secondary Metabolites of Fungal Origin: An Overview

Natural product chemistry owes its spectacular developments over the years to discoveries of novel molecules by organic chemists working on products of metabolism from the biological kingdom. Plants, animals and microorganisms have provided to mankind a large variety of biologically active compounds which have found diverse applications to his well-being in health and for curing diseases. Plants have provided dyes (indigo), perfumes (myrrh), spices (cinnamon), medicines (digitalis), poisons (hemlock), pharmaceutical agents (opium) and cosmetics (henna). Historically some macrofungi were known to be sources of hallucinogenic compounds, while the causal agent of a serious dangerous disease 'St. Anthony's fire' was known to be the ergot of rye caused by *Claviceps purpurea*.

If one carefully considers these compounds, it would be obvious that they represent species–specific and specialised molecules. These are not directly referrable to the essential metabolic functions associated with sustained growth processes of the concerned species. In other words, these compounds are "secondary" in origin and distinct from the primary products of metabolism responsible for synthesis of the various macromolecules required for the normal growth processes. **Bu'Lock** (*Adv. Appl. Microbiol*. 3:293–342, 1961) was the first to recognise the diversity of naturally occurring metabolites in the biological systems and which provide the organic chemist an exciting and challenging opportunity to

unravel their structure. Since these molecules were different and more complex than those involved in primary (general) metabolic pathways, he introduced the term 'secondary metabolites' to characterise them.

Microbial sources have proved to be goldmines of secondary metabolites and with the discovery of therapeutically useful compounds starting with penicillin, the search for diverse novel molecules with biological activity intensified. As is well known, the Actinomycetes have proved to be the most versatile group of microorganisms as far as synthesis of secondary metabolites are concerned. A very large number of antibiotics and other bioactive metabolites have been identified from these strains and the search continues worldwide in different laboratories to discover newer molecules. Recent years have seen a spurt in the focus of attention on screening fungal sources for novel secondary metabolites. In this chapter a general overview of fungal secondary metabolites is presented, primarily with a view to apprise the reader of the versatility of this group of organisms and the immense possibilities that exist for future discoveries. A comprehensive review on Bioactive fungal metabolites by **Pearce** (*Adv. Appl. Microbiol.* 44:1–79, 1997) may be consulted for greater details on this topic.

Over the years, the term 'secondary metabolites' has undergone considerable modification in its definition and interpretation with reference to the microbial systems. The metabolites were identified as those not directly connected with the growth processes and produced generally after the active growth phase (trophophase) is completed and with the onset of what is known as the idiophase (and secondary metabolites were also referred to as idiolites). It was also realised that the onset of enzymatic reactions triggering the secondary metabolite synthesis were favoured by depletion of one or more essential nutrients. In some cases the sensitivity of the secondary metabolism to the level of media ingredients such as phosphate was far greater than for metabolites involved in growth processes. Secondary metabolites have been regarded as having no obvious role to play in the economy of the producing species. They are derived from some of the

intermediates of primary metabolism and their formation represents an expenditure of energy (**Turner**. *Fungal Metabolites*, Academic Press, New York, 1971). Further, secondary metabolites are characterised by the following attributes: (a) they occur only in definite and taxonomically definable groups of organisms (b) they have numerous chemical groups which are absent in intermediary metabolism and possess highly complex structures arising through the action of multiple enzyme systems which are regulated by multiple genes.

The beneficial biological activities exhibited by various secondary metabolites make them invaluable candidates for a diversity of applications. Secondary metabolites of microbial origin have received greatest attention after the dawn of the antibiotic era and over the years several thousands of microbial metabolites of varied chemical complexities have been identified and evaluated for their usefulness to mankind. Bacteria, Actinomycetes and fungi have been subjected to extensive screening. Apart from antibiotic activity, secondary metabolites have been also successfully screened for applications in diverse areas. These include their application in agriculture as pesticides and herbicides, combating parasitic infections and in human therapy as enzyme inhibitors, immunomodulators and antihypertensives.

In the present discussion aimed at presenting an overview of fungal secondary metabolites, emphasis has been given to recent developments citing specific examples, with the intention of bringing out the leading trends for discovering new molecules. It does not give an exhaustive survey of the numerous microbial metabolites that have been described in literature. In discussing fungal secondary metabolites, many of the familiar bioactive compounds on which a large volume of literature is already available (e.g. penicillin, gibberellins, ergot alkaloids, steroid transformations, etc.) have not been included as relevant information on these well-known metabolites may be obtained from several excellent review articles available in literature.

A. Antibacterial Agents

Penicillin and Cephalosporin are the two most important and widely used antibacterial antibiotics produced by fungi. Recent advances in penicillin therapy including the production of semi-synthetic derivatives with wider antibacterial spectra have been reviewed extensively in literature. **Penalva** et al. (*Trends in Biotechnol*. 16:483–89, 1998) have reviewed the optimisation of penicillin biosynthesis in fungi and among the recent advances, isolation of the structural genes encoding the three main penicillin biosynthesis enzymes has stimulated the use of molecular approaches to analyze the currently employed industrial strains, which have been the products of over 50 years of strain and process improvement. *Aspergillus nidulans*, which is a penicillin producer and a genetic model system is also being studied for better understanding of the genetic regulation of the biosynthesis of primary and secondary metabolism and secretion of the end product.

From a biological point of view, it is interesting to note that there are several instances of the production of the same antibiotic by taxonomically unrelated microorganisms and this topic has been reviewed in detail by **Lechevalier** (*Adv. Appl. Microbiol*. 19:25–45, 1975). Benzylpenicillin is produced by several strains of *Penicillium* and *Aspergillus* species when phenylacetic acid or alpha phenyl acetamide is present in the medium. Under similar conditions penicillin biosynthesis also takes place in the dermatophyte *Trichophyton mentagrophytes* and thermophilic fungus *Malbranchea pulchella.* Other examples which may be mentioned include the production of gliotoxin, a sulphur containing antibiotic which is produced by *Penicillium, Gliocladium, Aspergillus* and *Trichoderma.* Oosporein, a red benzoquinone pigment with antibacterial and antifungal activity is produced by a wide range of taxonomically unrelated genera like *Oospora colorans, Acremonium, Beauveria, Chaetomium aureum* and *Phlebia mellea*. These examples bring into focus the need for utmost caution in the application of secondary metabolite production to gauge taxonomic relationships among fungi, which has been

advocated in certain situations. Presently the large number of useful antibacterial antibiotics have mainly originated from prokaryotes, primarily the Actinomycetes. The problem of drug resistance among pathogens to the currently used antibiotic molecules has necessitated the intensification of search for newer molecules and it is quite possible that future years may witness greater attention being paid to fungi as source for newer antibacterial compounds. From the present literature reports, there are relatively few novel and therapeutically promising antibacterial agents that have been identified from fungal sources.

B. Antifungal agents

Griseofulvin is a clinically important antibiotic used in the treatment of fungal infections and produced by fungi. Species of *Penicillium* such as *P.griseofulvum, P. janczewskii* and *P. nigricans* produce the an tibiotic besides other fungi like *Aspergillus versicolor, Nigrospora oryzae* etc. **Brian** et al. (*Trans. Brit. Mycol. Soc*. 29:173, 1946) described a substance produced by *Penicillium janczewskii* that would curl the growing mycelia of *Botrytis allii* which they named as "curling factor". This substance has been shown to be very effective in the treatment of fungal infections. Several publications and patents are reported in literature. The properties, biosynthesis and fermentation of Griseofulvin has been reviewed (**Huber** & **Tietz.** *Biotechnol. Indust. Antibiotics* (edited by **E.J. Van Demme**. 552–566, 1984). It has been used as an effective antibiotic against fungal infections, especially dermatomycosis of animals and humans by oral therapy (**Gentles,** *Nature* 182:476–77, 1958).

Recent years have witnessed an increase in the interest for identifying effective antifungal agents. This has been necessitated due to the expanding numbers of patients facing the risk of fungal infection following immunocompromisation for organ transplants, AIDS therapy etc. The development of resistance to the currently used therapies adds a further dimension to the problem. Antifungal compounds presently used act on targets which are also found in mammalian cells

and this may result in toxicity or an adverse drug reaction. It is imperative therefore to search for antifungal agents that are not toxic to mammalian cells. The polyene antifungal antibiotics produced by actinomycetes are currently used and one of the most extensively employed to treat systemic fungal infections is Amphotericin B. Fungi as sources for novel antifungal agents have been screened and among the many compounds isolated, the echinocandins and the Pneumocandins are highly potent and promising and are in clinical trials. The echinocandins are produced by *Aspergillus nidulans* and are lipopeptides which are inhibitory to β-1, 3 glucan synthesis. This compound has shown activity against both *Candida* and *Pneumocystis carinii*, which is the casual agent of pneumonia in immunocompromised (especially AIDS) patients. Intensive research has led to our present knowledge on the 'family' of echinocandin peptides which include the echinocandins, cilofungin, pneumocandins, aculeacins, mulundocandin, etc. Among the different echinocandins (B, C, D) identified, echinocandin B is the major species produced by some members of the *Aspergillus nidulans* and *A. rugulosus* groups. The haemolytic nature of echinocandin B has limited use in clinical medicine but isolation of analogues of echinocandins by enzymatic treatment (LY Compounds) with far less haemolytic activity have been identified. LY 12019, a semisynthetic derivative of echinocandin B which showed good in vitro and in vivo anti-*Candida* activity was reported to be at least twenty-fold less toxic than amphotericin B and therefore promises to be important in treating these types of infections (**Gordee** et al. *J. Antibiotics* 37:1054–65, 1984).

Schwartz et al. (*J. Antibiotics* 42:163–67, 1989) reported the isolation of a new antifungal lipopeptide L-671, 329 similar to echinocandin B from a mitosporic fungus, *Zalerion arboricola*. It has been reported that *Z. arboricola* also produces the pneumocandins which were effective against *Pneumocystis carinii* infections. Pneumocandin A the most important member of the group and its analogues have been widely investigated. Semisynthetic derivatives of Pneumocandin such as L-693, 989 were less haemolytic than the parent compounds. Several water soluble semisynthetic

derivatives having a wider spectrum of antifungal activity including against filamentous fungi like *Aspergillus flavus* and *A. fumigatus* and with relatively low haemolytic activity towards mouse and human erythrocytes have also been discovered. Aculeacins produced by *Aspergillus aculeatus* (**Mizuno** et al. *J. Antibiotics* 30:297–302, 1977) and Mulundocandins (**Mukhopadhyay** et. al. *J. Antibiotics* 40:281–89, 1986) are other compounds exhibiting similar antifungal activities.

C. Cholesterol Biosynthesis Inhibitors

One of the interesting developments has been the discovery of fungal compounds which are inhibitors of squalene synthesis which are also potent antifungal agents. Zaragozic acids and squalestatins independently discovered from Glaxo and Merck laboratories are both inhibitors of squalene synthetase and also find application in the lowering of serum cholesterol levels. Species of *Phoma* have been found to be promising producers of these compounds (**Dawson** et al. *J. Antibiotics* 45:639–47, 1992). The ability to produce zaragozic acids appears to be widely distributed among different fungal groups. **Bills** et al. (*Mycological Res*. 98:733–39, 1994) have given an account of the distribution of zaragozic acids (squalestatins) among fungi and list several genera besides *Phoma* including *Leptodontium elatius, Sporomiella intermedia, Curvularia lunata, Drechslera biseptata, Pseudodiplodia* sp. *and Amauroascus niger*. The zaragozic acids which are picomolar competitive inhibitors of squalene synthesis have been extensively reviewed (**Bergstrom** et al. *Proc. Natl. Acad. Sci.* USA 90:80–84, 1993, *Ann. Rev. Microbiol*. 49:607–39, 1995). As a result of screening fungi for hypocholesterolemic agents, very useful and clinically important drugs have been developed. The most important is Lovastatin which was first reported by Merck from *Aspergillus terreus*. Related compounds were reported by the research groups at Beecham laboratories from *Penicillium brevicompactum* and *P. citrinum*. The product from *P. brevicompactum* named as Compactin also had antifungal

activity. (**Brown** et al. *J. Chem. Soc. Perkin Trans*. 1:1165–70, 1976). At the Sankyo laboratories in Japan, Endo and collaborators have been working with *Monascus ruber* and have identified several inhibitors of cholesterol biosynthesis which they have named as Monacolins (Monacolin A, J, K, L, etc.) which have been published in a series of papers in the *J. Antibiotics, Japan* (**Endo** et al. *J. Antibiotics* 32:852–54, 1979, 33:334–36, 1980, 38:321–27, 1985, 38:420–22, 1985, 39:1670–73, 1986). These compounds inhibit the rate limiting step in cholesterol biosynthesis, HMG CoA Reductase and they are used clinically to reduce the cholesterol levels.

D. Immunosuppressive Compounds

The best known and widely used immunosuppressive compound of fungal origin is Cyclosporin, a metabolic product of *Tolypocladium inflatum*. Originally discovered as an antifungal agent from this fungus besides *Cylindrocarpon lucidum* (**Dreyfus** et al. *Eur. J. Appl. Microbiol. Biotechnol*. 3:125–33, 1976), it was subsequently discovered to have excellent immunosuppressive activity and useful for treatment following organ transplants. The Cyclosporins represent a group of biologically active secondary metabolites from *Tolypocladium* as well as other genera such as *Beauveria, Fusarium, Paecilomyces* and *Verticillium*. The main component in normal fermentation broths is Cyclosporin A and is used as a powerful immunosuppressant in organ and bone marrow transplatations. Over 25 congeners of Cyclosporin have been identified and isolated from fermentation broths besides cyclosporin A. All the natural Cyclosporins are neutral cyclic oligopeptides composed of eleven aminoacids, several of which are *N*-methylated and one or two belong to the D-series. The course of Cyclosporin biosynthesis can be strongly influenced by an exogenous supply of amino acid precursors to the fermentation medium. For instance, L-alpha-aminobutyric acid, L-alanine, L-threonine, L-valine and L-norvaline get incorporated preferentially to enhance yields of the corresponding cyclosporin A, B, C, D and G, respectively (**Kobel** & **Traber**.

Eur. J. Appl. Microbiol. Biotechnol. 14:237–40, 1982, **Traber** et al. *J. Antibiotics* 42:591–97, 1989).

Mycophenolic acid is another example of a fungal-based immunosuppressant. It was initially discovered from *Penicillium brevicompactum* as early as 1896 and subsequently found in a number of other penicillia. Its structure was described in 1952. In vivo, its mode of action has been to inhibit the biosynthesis of certain precursors of nucleic acids and thereby inhibit the proliferation of cells which are involved in the immune response. Syntex/Roche have developed Mycophenolic acid mofetil as an immunosuppressive drug which has been approved for use following kidney transplantation. Mycophenolic acid represents an example of finding new applications for a classical metabolite known for a long time and this brings into focus the importance of screening known metabolites for newer applications besides searching for new chemical structures and molecules.

A metabolite with immunosuppressive activity was discovered by **Fujita** et al. (*J. Antibiotics* 47:208–13, 1994) from *Isaria sinclarii*. This compound designated ISP-1 was reported to be several-fold more potent than cyclosporin A in suppressing lymphocyte proliferation in vitro and in in vivo bioassays.

E. Antiviral and Anticancer/Antitumor Activities

Search for antiviral activity is planned by having an understanding and knowledge of the biochemical events that predispose the onset of viral infection. For example, during HIV replication, a series of complex events including specific binding of viral RNA with viral and host proteins is involved. The viral protein has a regulatory role in the transport of viral RNA into host cytoplasm and it is envisaged that blocking this interaction could yield antiviral compounds. By developing sophisticated techniques, scientists at the Bristol Myers Squibb laboratories identified from *Trichoderma harzianum* compounds designated Harziphilone and Fleephilone, which

exhibited such inhibitory activities (**Qian-Cutrone** et al. *J. Antibiotics* 49:990–97, 1996).

Anticancer and antitumor activity of diverse mould metabolites with varying degrees of effectiveness in combating the disease and different levels of toxicity to the normal healthy cells have been reported in literature. Novel cell cycle inhibitors termed Tryptostatins have been isolated from *Aspergillus flavus* (**Cui** et al. *J. Antibiotics* 48:1382–84, 1995) while compounds inhibiting metastasis have been identified from the mitosporic fungus *Nattrassia mangiferae* (**Chu** et al. *J. Antibiotics* 48:329–31, 1995).

Biochemical events predisposing cells before the onset of cancer have been analysed and inhibitors for some of the key events at the enzymic level have been projected for their anticancer and antitumor potential. For example the Ras gene is known to be associated with many cancers and before cells get transformed it is known to bind to GTP. The enzyme farnesyltransferase is involved in the process and the inhibition of this enzyme could be a potential target for anticancer activity. A number of fungal metabolites including gliotoxin, andrastins, kurasoins and fusidinol have exhibited this activity. Kurasoins are products of *Paecilomyces* (**Uchida** et al. *J. Antibiotics* 49:932–34, 1996) while Fusidienol has been isolated from *Fusidium griseum* (**Singh** et al. *Tetrahedron Lett*. 35:4693–96, 1994).

Taxol is a promising anticancer drug obtained from the Pacific yew plant (*Taxus brevifolia*) and being a slow-growing tree, raw material availability is always scarce. The discovery that an endophytic fungus *Taxomyces andreanae* associated with the *Taxus* bark can produce taxol by fermentation and independent of any inputs from the yew plant has opened a new area of research and development for this anticancer drug (**Stierle** et al. *Science* 260:214–216, 1993), **Stierle** et al. *J. Natural Prod*. 58:315–24, 1993, *Strobel et al*. WO93/21338 (PCT/US93/03416). A patent application by Novopharm, Canada has claimed isolation of taxol producing microorganism from the leaves, branches, twigs and bark of the ornamental yew shrub *Taxus hickii* and used in

fermentation to produce a biomass containing taxol. One such taxol-producing isolate with significant yields has been identified as *Alternaria alternata*. **Strobel** et al. (*Microbiology* 142:435–40, 1996) isolated a taxol-producing strain of *Pestalotiopsis microspora* as an endophyte of the Himalayan yew (*Taxus wallichiana*) and obtained yields of taxol from the broth as well as the mycelium equivalent to 45–65 µg per 500 ml medium.

F. Anti-Invertebrate/Insecticidal Compounds

Fungal metabolites active against protozoa, nematodes and insects have received widespread interest and attention. Protozoan infections like amoebiasis due to *Entamoeba histolytica* and coccidiosis in chick caused by *Eimeria tenella* are among the most important protozoan diseases for which cures have been sought among microbial metabolites. Antiamoebin, a peptide isolated from *Emericellopsis synnematicola* and related species by **Thirumalachar** (*Hindustan Antibiotics Bull*. 10(4): 287–289, 1968) has shown high activity against these pathogens and has been demonstrated to be effective in vivo in treating these diseases. This antibiotic has also been shown to be effective against other protozoan parasites like *Trichomonas vaginalis* and *Trypanosoma evansii*. It also showed activity against plant pathogenic nematodes, and was effective against intestinal parasitic nematodes affecting cattle. **Tabata** et al. (*J. Antibiotics* 48:53–58, 1995) isolated Fudecalone from a *Penicillium* which was a terpene type metabolite which was inhibitory to *Eimeria tenella*. One of the most widely used antiparasitic compound is the actinomycete metabolite Avermectins.

Nematode-trapping fungi and their possible role in the control of plant-pathogenic nematodes destructive to crop plants has also received attention and predaceous fungi like *Arthrobotrys*, *Dactyella* and *Dactylaria* have been studied mycologically and as possible biocontrol agents. From *Arthrobotrys oligospora*, Oligosporons have been identified which are nematicidal metabolites (**Anderson** et al. *J.*

Antibiotics 48: 391–98, 1995). **Stadler** et al. (*J. Antibiotics* 48: 261–66, 1995, 48: 267–70, 1995) studied Ascomycetes for metabolites affecting nematodes and identified a series of active compounds like Lachnumon, Lachnumol A etc. *from Lachnum papyraceum*.

Several fungal pathogens of insects have been investigated for biocontrol among which species of *Beauveria, Metarrhizium, Nomuraea* etc. have received the maximum attention. Insecticidal microbial metabolites have also been widely studied among which the polyoxins and nikkomycins produced by Actinomycetes are useful for control of insects by virtue of their ability to inhibit chitin synthesis. Chitin synthesis inhibitors as well as chitin degrading enzymes (chitinases) have potential both as insecticidal metabolites and antifungal agents. In recent years there has been considerable interest in exploiting compounds with ability to interfere with chitin metabolism for applications related to combating fungal infections as well as for controlling insect populations. It has been observed that fungal sclerotia which help the fungi to tide over unfavourable conditions in the natural environment elaborate compounds which are insect repellants or insecticidal. A group of compounds known as Aflavines have been identified from sclerotia of several *Aspergillus* species as well as from the ascostroma of *Eupenicillium crustaceum*.

Cyclodepsipeptides which are toxic to insects and other invertebrates have been identified from several entomogenous fungi and include Beauvericin from *Beauveria bassiana* (**Hamill** et al. *Tetrahedron Lett*. 49:4255–58, 1969), Bassionalide from *B.bassiana* and *Verticillium lecanii* (**Suzuki** et al. *Tetrahedron Lett*. 57:2167–2170, 1977) and Destruxins from *Metarrhizium anisopliae* (**Suzuki** et al. *Agr. Biol. Chem*. 24:813–16, 1970). From *Isaria felina* three insecticidal cyclodepsipeptides were identified by **Baute** et al. (**J. Antibiotics** 34:1261–65, 1981).

Chitinases hydrolyse chitin into oligomers of N-acetylglucosamine. At present only a few inhibitors of chitinases such as Allosamidin from *Streptomyces* and

styloguanidine from sponges have been characterised. While commercial applications of chitin synthesis inhibitors as fungicides and insecticides have been under intensive study and development, chitin degradation inhibitors have not been sufficiently investigated. An inhibitor of chitinase could be expected to inhibit the moulting of insects and prevent their maturation to the adult reproductive stage. **Omura** et al. (*J. Antibiotics* 53:603–608, 2000), discovered a new cyclic peptide chitinase inhibitor from the cultured broth of *Gliocladium* sp. which they designated Argifin. This has been claimed to be the first chitinase inhibitor produced by fungi with a possible mechanism of action different from the allosamidins.

G. Other Bioactive Metabolites

Alternaria alternata produces a metabolite termed Tentoxin and this is a phytotoxin with herbicidal activity (**Lax** & **Shepherd** in ACS Symposium Ser. 380 Ed. **H.C. Cutter** p.24–34, 1988). Other examples of fungal-based herbicide include Cornexistin from *Paecilomyces varioti* which is herbicidal against grass and broad-leafed species (**Nakajima** et al. *J. Antibiotics* 44:1066–72, 1991). **Tanaka** et al. (*J. Antibiotics* 49:1056–59, 1996) isolated Dechlorogeodin with herbicidal activity from a soil isolate of *Chrysosporium*.

Search for fungal metabolites having vasodilator activity and prevention of platelet aggregation has led to the discovery of some interesting compounds. **Takase** et al. (*J. Antibiotics* 37:1320–23, 1984) isolated a new alkaloid Amauromine from culture broth of *Amauroascus* with vasodilating activity. It had low toxicity to mice and is classified as a novel calcium entry blocker. **Ando** et al. (*J. Antibiotics* 41:25–30, 1988) isolated a novel antihypersensitive and platelet aggregation inhibitory agent designated Vinigrol from cultured mycelia of *Virgaria nigra*. Vinigrol decreased arterial blood pressure of anesthesised normotensive rats when administered intravenously. Vinigrol also inhibited platelet activating factor and epinephrine induced platelet aggregation.

Endo et al. (*J. Antibiotics* 36:203–207, 1983) identified Mutastein from culture broths of *Aspergillus terreus* as a new

inhibitor of adhesive glucan synthesis by the dental caries inducing *Streptococcus mutans*. Water insoluble glucans synthesised from sucrose are known to be a major virulence factor for induction of dental caries in man and animals. These glucans are synthesised by the action of glucosyl transferases and the glucans form a firm coating on the tooth surface. Mutastein is a heat-stable protein which inhibited the glucosyl transferase and caused interference in the glucan formation. **Murakawa** et al. (*J. Antibiotics* 40:394, 1987) identified several fungal strains that produced a specific inhibitor of adhesive glucan synthesis by *Streptococcus mutans* including species of *Cladosporium, Leptographium, Sporomiella*, etc. However the highest activity, more than four times that of *Aspergillus terreus* was observed in the case of a strain of *Drechslera erythrospila*.

H. Mycotoxins

Many fungi produce toxic compounds which adversely affect human and animal systems and these toxic metabolites are termed Mycotoxins. These are also secondary metabolites, which have been studied for understanding their biosynthesis. This knowledge has been used to control their formation in food materials and feeds. Aflatoxin produced by *Aspergillus flavus* is one of the most stable carcinogenic compounds produced in groundnut and a variety of other food materials during post-harvest operations and storage. Other examples of mycotoxins include Zearalenone from *Fusarium graminearum* (telomorph: *Gibberella zeae*) which causes vulvovaginitis and infertility in cattle and pigs. Trichothecenes are another group of toxins produced by *Fusarium tricinctum*, *Fusarium sporotrichoides* and *Fusarium poae* etc. and these toxins cause alimentary toxic aleukia in farm animals as well as humans. Slaframine is a toxin from *Rhizoctonia leguminicola* causing excessive salivation in cattle referred to as "Slobber Syndrome". Islandicin and Luteoskyrin are carcinogenic toxins from *Penicillium islandicum*, the cause of hepatitis in humans.

The subject of mycotoxins is vast and what has been presented are just a few examples of some of the important toxins produced by fungi. For more detailed information, review articles on the different toxins may be referred.

Discussion and Conclusions

From the discussion and examples cited, it is evident that the fungi are promising and potent resources for diverse secondary metabolites of value. Intensification of the search and extending it to include hitherto neglected or unexplored taxa could prove beneficial. The success of the programme, however, will depend on several factors that go into designing a successful screening strategy. These include incorporation of new detection methods and new source microorganisms, devising sensitive, selective and yet rugged assay systems unaffected by interference from other broth constituents, ability to recognize known and undesirable compounds early and organising a co-ordinated inter-disciplinary approach for high through-put screening. (**Franco** & **Coutinho**. *Critical Rev. Biotechnol*. 11:193–276, 1991). From a mycological point of view, the formulation and standardisation of the fermentation conditions favourable for the elaboration of the secondary metabolite(s) is of the utmost importance and manipulation of the fermentation conditions remains largely empirical. Ability to visually recognise the presence of the desired bioactive metabolites is very helpful. For example, observation of induced morphological abnormalities in fungal and yeast cells would be useful in detecting antifungal metabolites. (**Gunji** et al. *Agric. Biol. Chem*. 47: 2061–69, 1983). **Steinberg** et al. (*J. Antibiotics* 38:1401, 1985) described the development of a rapid, sensitive microbiological process using a 'dim' variant of *Photobacterium leiognathi* to detect the potent antitumour compounds that bind to or affect DNA and cause stimulation of bioluminescence. Such bioluminescence stimulation in this strain has been described as a test for genotoxic compounds.

As pointed out by **Demain** in an article entitled "Microbial Natural Products: Alive and well in 1998 (*Nature Biotechnol*. 16:3–4, 1998), natural product research is at its highest level as a consequence of unmet medical needs and on the remarkable diversity of natural structures and activities. As the search intensifies and advances in scientific knowledge and techniques take place in future years, one can surely envisage the significant role that fungi and mycologists would contribute to the discovery process for novel and useful bioactive molecules.

CHAPTER 9

Fungal Resources for Industrial Enzyme Technology

Industrial enzymes occupy an important niche in the area of microbial fermentation technology and the search for novel enzymes suited to diverse applications from different microbial sources has been on the increase. Bacteria, Actinomycetes and fungi have been successfully employed in optimising fermentation processes for the manufacture of large quantities of enzymes for applications in the food, pharmaceutical, textile, leather, detergent and many other industries. Enzyme applications for mankind's benefit predate the technical knowledge on the nature of the enzymes. For example, starch saccharification in the traditional brewing was carried out by the use of indigenous enzymes in malted barley, while in the oriental fermented foods, microbial enzymes native to the substrates were responsible for the fine flavour changes effected by the fermentation. Use of calf rennet for cheese making or the use of papain for the tenderising of meat during cooking are other well-known examples of enzyme applications in traditional food processing.

The majority of microbial enzymes produced on a commercial scale are extracellular whereas some of the commercially used enzymes like glucose isomerase from actinomycete strains are intracellular requiring extraction from the cells after fermentation or used by suitable whole-cell immobilisation techniques. Extracellullar secretion of the

enzyme offers the advantage of obtaining large quantities in a relatively pure state free from other cellular proteins associated with the organism and can be easily processed by filtering off the cells and isolating the enzymes from the cell-free filtrate. Most of the enzyme fermentations are carried out in submerged culture. However recent trends indicate that greater attention is being paid to standardise enzyme technologies based on solid-state fermentations.

Fungi have received considerable attention as sources of large-scale production of industrially useful enzymes. From the classical process for the manufacture of 'Taka Diastase' on wheat bran koji developed by **Jokichi Takamine** using *Aspergillus oryzae* (US Patent 525823,1894), fungal-based enzyme technology has come a long way and today the emphasis is on exploring naturally occurring fungal strains for a variety of enzymes, notably hydrolases of carbohydrates, proteins and fats. Amylase, Protease, Pectinase, Cellulase and lipase are among the diverse enzymes manufactured by fungal fermentation. Attempts to find substitutes for useful enzymes originating from animal sources through fungal fermentation have also been sucessful. For example the production of milk clotting enzymes suitable for cheese making from mucoralean fungi *Rhizomucor miehei* and *R. pusillus* and more recently the cloning and expression of Chymosin as a heterologous protein in *Aspergillus niger* (discussed in an earlier chapter) are significant developments from an industrial point of view. Compared to bacterial fermentations in which the process technology involves the use of sophisticated equipments for getting clear filtrates from the colloidal broths, fungal broths can be easily filtered by filter press or similar simple equipment saving considerable investment costs for equipment.

Hydrolytic enzymes are produced by microorganisms to break down complex naturally occurring nutrient molecules and assimilate them for metabolism and growth. For example when inoculated on a starch-containing medium, amylases are secreted to degrade the starch molecules to glucose and utilise it. Likewise protease secretion is induced to degrade complex proteinaceous nutrients into amino acids before taking them

up for metabolism and cellular biomass build up. Overproduction of enzymes and secreting them in quantities far beyond the amounts necessary for the hydrolysis of complex nutrient substrates is a trait associated with select number of species and in a sense these secreted high levels of enzymes can also be regarded as secondary metabolites. Often the levels of enzyme activity reach a peak level after much of the component substrate(s) has been substantially degraded. Fungi have been found to be extremely efficient secreters of soluble protein and under optimised conditions of fermentation, mutant strains secrete up to 30 grams per litre of extracellular protein. In the strains selected for enzyme fermentations, the desired enzyme constitutes the only component or at least forms the major ingredient of the secreted protein with high specific activities. It is this trait of high-level protein secretion besides their eukaryotic nature that has made the fungi as favourite hosts for heterologous expression of high value mammalian proteins for manufacturing by fermentation.

Factors Regulating Extracellular Enzyme Production

All important industrial enzymes are hydrolases and in the natural environment microorganisms catalyse the breakdown of complex substrates through hydrolytic enzymes to derive essential nutrients for metabolism and growth. Because their function is remote and once released from the cell they cannot be controlled by the cell, the extracellular enzymes tend to be very stable and also tend to be produced in relatively large amounts. This means that even wild strains isolated from the natural environment are potentially capable of producing significant levels of enzymes for commercial manufacture**. Ryu et al**. (*Biotechnol. Bioeng*. 21: 1887–1903, 1979) analysed experimental data on cellulase production in a two-stage continuous culture. According to their estimates with specific activity of cellulase of 0.6 unit per mg protein and for an yield of enzyme of 1610 units per gram nitrogen consumed, it can be

calculated that approximately 6% of the total nitrogen is contributed to the enzyme molecule. The higher stability of extracellular enzymes and their broad tolerance to pH and temperature variations make them very useful for practical commercial applications. Complex regulatory mechanisms control biosynthesis and secretion of these enzymes. They are induced by low levels of the hydrolysis products of the polymers they hydrolyse and sometimes the polymers themselves seem to function best as inducers. Sometimes compounds, which are not substrates for the enzyme, can also act as alternate inducers. For example, cellulase is induced by cellobiose or lactose or even by a disaccharide sophorose (**Mandels** et al. *J. Bact*. 83:400–408, 1962). Enzyme production is subject to catabolite repression. While assimilable low molecular weight nutrients are available, the cells will grow without producing extracellular hydrolases and the production machinery will get activated only when the assimilable nutrients are exhausted. For example in a medium containing glucose and cellulose, cellulase induction can be observed only after exhaustion of supply of the readily metabolisable glucose. Extracellular enzymes account for about two thirds of total world market for industrial enzymes. Certain properties of enzymes necessitate special consideration in the design of suitable media for their production. These relate to the phenomena of induction, catabolite repression, product inhibition and in the case of extracellular enzymes, protein release mechanisms. In some cases the main carbon source can also serve as an inducer, as in the case of cellulase induction by cellulose. Use of analogues or other compounds which can serve as inducers are however too expensive for practical use in the commercial manufacture of these enzymes. Mutation at a regulatory site to eliminate the requirement for an inducer, elimination of the ability of the operator gene to bind a repressor molecule or prevention of the formation of a repressor molecule are some of the molecular mechanisms that can lead to the development of the so-called 'constitutive' mutants producing significant levels of the enzymes in the absence of an inducer or even in media containing compounds exerting repression of enzyme synthesis. Catabolite repression is a process in which

synthesis of key enzymes in a metabolic sequence are suppressed when an easily metabolised carbon source is present. Glucose commonly represses the formation of catabolic enzymes and this is termed the 'glucose effect'. This type of repression may be prevented in the design of the fermentation medium by avoiding the use of repressive sources of carbon. Providing the metabolic carbon in the form of slowly metabolisable compounds like starch or lactose rather than glucose would largely overcome catabolite repression.

An alternative approach would be to feed the potential repressive substrate slowly into the fermentation broth so that its concentration during fermentation is kept at a low and non-repressive level. Repression of enzyme synthesis by end-products is another factor to be considered as seen in the case of protease synthesis repression by amino acids. The design of the fermentation medium must take into account the potential of such repression mechanisms to exert deleterious effects. For example, **Tomonaga** (*J. Gen. Appl. Microbiol.* 12:267–76,1966) designed a fermentation medium and operated the fermentation conditions under sulphate limitation since protease synthesis in *Aspergillus niger* was strongly influenced by the levels of sulphate in the medium. An alternative approach would be to prevent the formation of the end-products which are inhibitory by incorporating inhibitors of the end-product metabolic pathway into the medium.

Increased secretion of extracellular enzymes has been observed under the influence of surface active agents. In presence of 0.1% Tween-80, fifteen to twenty-fold enhanced secretion of cellulase, invertase, β-glucosidase and xylanase was recorded by **Reese** (*Biotechnol. Bioeng. Symp.* 3:43,1972). The effect may be due to modification of cell membrane by surfactants to increase its permeability and "leakiness" to the enzyme. For optimising process technology, the composition of the medium is important and must take into account the cost and availability of the raw materials and also the ease of downstream processing including filtration and post-harvest processing of the effluents. The medium optimisation should

also be related to the control of growth morphogenesis to favour filamentous or pellety growth and in relation to the levels of enzyme productivity. Also the medium optimisation for development of an economically viable process proceeds along with continuous strain improvement programmes.

Enzyme fermentations have been carried out by submerged culture in batch, fed-batch or continuous mode of operation. Batch fermentations in which the nutrient addition is carefully monitored and dosed have yielded the most promising results. The principal factors which influence the course of an enzyme fermentation are temperature, pH, dissolved oxygen tension which is itself a function of the aeration rate, agitation rate and gas phase pressure applied to the fermenter system. For example temperature profiling has been found to be beneficial in some cases. **Tangnu** et al. (*Biotechnol. Bioeng.* 23:1837–49, 1981) found in *Trichoderma reesei* mutant RUT-C-30, optimum cellulase production was obtained when the temperature was maintained at 31°C during the first 48 hours while the remaining period of the fermentation was carried out at 28°C. With the availability of instrumentation control facilities as well as online monitoring of fermentations, accurate assessment of different variables in the fermentation parameters has become possible and enhanced enzyme productivities could be standardised through careful studies to arrive at the optimal parameters with reference to individual production strains.

Hydrolytic Enzymes

A. Lipases Lipases as fermentation products have received considerable attention and several fungi are identified as source materials for such fermentations. These include species of *Aspergillus* such as *A. niger* and *A. luchuensis*, *Rhizopus delemar* and *R.arrhizus*, *Mucor lipolyticus, Geotrichum candidum* and *Humicola lanuginosa.* Lipases are a complex of enzymes with varied substrate specificities and in fermentations more than one lipase may be produced by a fungus, which may have different genetic origins or may be produced as a result of modifications to a single enzyme

protein. *Penicillium crustosum*, for example, produced two lipases with different relative activities towards tributyrin and olive oil (**Oi** et al. *Agric. Biol. Chem*. 31:1357,1967). Lipase production has been studied in both submerged and SSF fermentation conditions and the choice of the method varied with the strains employed. The presence of an inducer like triglyceride, long chain fatty acid ester or free fatty acid was favourable for lipase synthesis. In *Candida cylindracea*, extensively studied for its lipase activity, highest titres were obtained when a sterol and a fatty acid or its derivative were both present (**Ota** et al. *Agr. Biol. Chem*. 32:1476–78,1968). In other cases, requirement for an oil or fatty acid derivative did not appear to be absolute and in a few instances marked decrease in lipase production was observed when oil was incorporated. It has been reported in many fungi that the production of lipase has shown little sensitivity to the nature of the carbohydrate used and on media containing simple sugars, lipase yields were independent of the type of sugar used. In the light of recent advances and newer applications of lipases for specific objectives, search for lipases with specific properties to meet the requirements and optimisation of the fermentation conditions to obtain the desired lipase in high yields need to be investigated and standardised.

B. Starch Degrading Enzymes Fungal alpha amylases differ from the bacterial amylases in the spectrum of sugars produced from starch yielding a product containing mainly maltose and some glucose. While *Bacillus* amylases are highly thermostable and act in the endo mode to release dextrins, the fungal alpha amylases are also less thermostable. Alpha amylase from *Aspergillus oryzae* was the first microbial enzyme to be manufactured for sale and was made by solid state cultivation for many years (**Takamine**, US Patent 525823, 1894). *A. oryzae* is almost universally used as the source of commercial fungal alpha amylase. **Yabuki** et al. (*Appl. Environ. Microbiol*. 34:1–6,1977) studied rapid induction of alpha amylase by non-growing mycelia of *A. oryzae* and observed maltose was giving the best activity followed by alpha-methyl β-D-glucoside and starch. However natural media containing starch or brans as sources of growth

yielded higher enzyme titres compared to maltose, sugars or dextrins. In their studies, **Meyrath** and **Bayer** (*Proc. FEBS Meet Int. Clin. Enzyme*. 61:331,1980) reported that alpha-linked glucose substrates favoured amylase production compared with monosaccharides and the production is enhanced by excess ammonium ions. They also claimed that solid state cultivation gave higher productivity of the enzyme compared with submerged culture. In addition to *A. oryzae,* strains of *A. niger* have also been widely investigated for alpha amylase production. **Heidy-Hansen** and **Aunstrup** (European Patent 138428, 1985) have described the production of an acid stable alpha amylase from *A. niger*. By mutation and selection of suitable cultural conditions, suppression of the accompanying amloglycosidase and transglucosidase activities was achieved facilitating the use of the enzyme in the production of high maltose syrups. For complete hydrolysis of starch to glucose the combined action of alpha amylase and amyloglucosidase is necessary. Amyloglucosidase (glucoamylase) attacks the non-reducing chain ends of starch and release -glucose. It also slowly attacks the alpha-1, 6 branch points of amylopectin so that almost quantitative yields of glucose can be obtained from starch subjected to thinning by alpha amylase treatment. Strains of aspergilli notably *A. niger* and species of *Rhizopus* such as *R. delemar* are rich sources of amyloglucosidase. Large-scale manufacture of alpha amylase and amyloglucosidase has enabled the manufacture of glucose from starch through enzymatic hydrolysis worldwide completely replacing the earlier technologies based on the use of acid for effecting the hydrolysis. The enzymes from *R. delemar* and *A. niger* have been characterised as exo-alpha 1,4 glucohydrolases and a strain of *A. niger* designated NRRL 337 has been widely used in the commercial manufacture of amyloglucosidase. Use of transglucosidase negative mutants of *A. niger* have made the process technology even more suited for large scale applications. Amyloglucosidase production is mostly carried out by submerged culture on media containing maize starch with or without pretreatment with bacterial alpha amylase supplemented with cornsteep liquor and inorganic salts. Multiple forms of amyloglucosidases differing in their

isoelectric points have been identified and separated from commercial samples of *A. niger* amyloglucosidase.

C. Cellulolytic and Related Enzymes Many microorganisms are capable of digesting cellulose and these include bacteria (*Cellulomonas, Cytophaga)*, Actinomycetes (*Thermomonospora)* and moulds (*Chaetomium, Trichoderma, Aspergillus and Penicillium)*. However only select fungi have been shown to be capable of secreting significant levels of extracellular cellulase enzymes suitable for enzymatic hydrolysis of cellulose to produce glucose. For complete hydrolysis of crystalline cellulose to glucose, it is usual for three types of enzymes to act synergistically, viz. endo β-1,4 glucanases which attack the bonds at random position in the cellulose chain, exo-β-1,4 glucanases which split cellobiose residues from the chain ends and β-glucosidases which split cellobiose to yield glucose. Multiple isoenzymes of endo and exoglucanases have been studied and characterised from cellulolytic fungi like *Trichoderma* and *Aspergillus*. Because cellulose is the most abundant and renewable natural carbohydrate of plant origin, there is a lot of interest in its breakdown to yield sugars for food, chemical feedstocks and fermentation. In particular, fermentation of cellulose-based feedstocks to produce liquid fuels has received considerable research. Development and optimistic projections of unlimited supply of renewable liquid fuels like ethanol to meet the demands in an era of depleting petroleum reserves continue to draw the attention of biotechnologists worldwide. A huge volume of research into cellulases and their potential applications has been undertaken during the last three decades. Considerable literature on various aspects of cellulose biotechnology is available. As yet however commercial-scale application of cellulases for conversion of cellulosic residues to ethanol have not met with commercial and economic success.

A strain of the fungus *Trichoderma*, isolated from the US Army equipment deteriorating in storage has received considerable attention. This fungus was selected as producer of an active complex of cellulases capable of digesting crystalline cellulose and was originally identified as *Trichoderma viride*. In subsequent investigations, the strain

has been placed under a new species, designated *Trichoderma reesei,* named in honour of the pioneering cellulase researcher **Dr Elwyn T. Reese.** The work on this fungus including isolation of a large family of improved cellulolytic mutants and also the construction of a pilot plant where the enzyme was used to hydrolyse cellulose to glucose has been comprehensively reviewed by **Reese** and **Mandels** (*Ann. Rep. Fermentation Process* (edited by G.T. Tsao 7:1–20, 1984). Several other fungi have been reported as sources of extracellular cellulase complex of enzymes and these include *Penicillium funiculosum, Sclerotium rolfsii* and *Talaromyces* sp. Higher levels of β-glucosidase secretion compared to *T. reesei* has been reported in case of *Scytalidium lignicola* (**Desai** et al. *J. Ferm. Technol.* 60:117–24, 1982) and *Penicillium funiculosum* (**Lachke** et al. *Biotechnol. Lett.* 5:649–52, 1983). Economically viable process development for enzymatic conversion of the abundantly available natural cellulosic substrates to fermentable sugars has so far remained an unfulfilled dream. In enzymatic digestion, the complex structure of the cellulosic materials in which the cellulose is bound to lignin and may have low accessibility to the enzyme, dictates costly mechanical pretreatment and also alkali treatment to make the cellulose less crystalline and susceptible to the action of enzymes. Also the very low rate at which cellulase enzymes catalyse the digestion of natural cellulose is a major hurdle in the development of practical and viable technology. It is worthwhile to note that the activity of a pure cellulolytic enzyme on a natural substrate is less than one mole glycosidic bond cleaved per minute per mg protein, which is about 1% of the corresponding rate of attack of amylolytic enzymes on starch.

Recent years have seen much interest in the development of cellulases stable to and active under alkaline pH conditions for potential applications in the detergent and textile industries. Alkalophilic *Bacillus* strains studied by the Kao Corporation in Japan produce an alkaline cellulase and such cellulase has been incorporated in commercial detergent named 'Attack'. The cellulase introduced in laundry detergents exhibit fabric softening and colour brightening properties besides removing

soil. Cellulases active at alkaline pH also find extensive application in the manufacture of denim jeans to produce the 'stone washed' effect and also 'biopolishing' of fabrics to achieve increased smoothness and softness of the finished product. Fungal sources of alkaline cellulase have not been extensively investigated. **Kang** and **Rhee** (*Biotechnol. Lett*. 17:507–12, 1995) identified an alkalophilic cephalosporium having carboxymethyl cellulase activity optimally between pH range of 7.5 and 9.5 and retaining 80% of its activity at pH 11.0 for 24 hours. The enzyme was also compatible with the components of laundry detergents. According to a report on Industrial enzyme technology (In *Trends Biotechnol*. 14(6): 177–178, 1996), cloning of the individual components of the *Trichoderma* cellulase complex (endoglucanase, cellobiohydrolase and β-glucosidase) has led to an improved understanding of cellulase activity related to the molecular structures of different cellulose polymers and the development of "customized cellulases for specific 'stone washing' process".

Natural cellulosic materials contain other carbohydrate polymers particularly the hemicelluloses which may also be hydrolysed by the cellulase. Beta glucans present in barley comprise glucose units linked by β-1,3 as well as β-1,4 linkages and in the brewing industry, the β-glucans impair the filtration of the wort. Enzymatic treatment with cellulases or -1,4 glucanases is beneficial in the speeding up of the filtration. Culture filtrates of cellulolytic fungi may have adequate levels of β-glucan hydrolysing activity to be of practical and commercial significance.

Xylans are xylose polymers, which are present in substantial amount along with cellulose, especially in the tropical plant residues. Many of the cellulase secreting fungi also elaborate xylan degrading enzymes, especially when the fermentation media contain natural agro-residues such as wheat bran, which are also rich in hemicelluloses. Enzymatic hydrolysis of xylans to xylose and its fermentation to ethanol by xylose-fermenting yeasts like *Pachysolen tannophilus* or fungi like *Neurospora* (*Monilia*) *crassa* has also received considerable research attention but so far has eluded translation on to practical technologies for commercial ethanol

production. In recent years there has been considerable interest evinced in discovering xylanase enzymes, which are not associated with high levels of cellulase activity. Such enzymes are recognised for their potential as 'environmentally friendly' alternatives for effective bleaching of paper pulp without the use of toxic chlorine compounds and without adversely affecting the quality of the paper pulp. Several alkalophilic *Bacillus* strains as well as actinomycetes secreting cellulase-free xylanases have been identified. Production and application of cellulase-free xylanases in the paper industry has been reviewed by **Srinivasan** and **Rele** (*Indian J. Microbiol*. 35:93–101,1995, *Current Sci*. 77:137–42,1999). Xylanases from fungal sources which are cellulase-free and active at high alkaline pH have not been much explored. At the National Chemical Laboratory, Pune **Bansod** et al. (*Biotechnol. Lett*. 15:965–70,1993) isolated a *Cephalosporium* strain (NCL 87-11-9) which grew well at pH above 9.0 and secreted significant levels of cellulase-free xylanase active at high alkaline pH and this was the first report of such an enzyme. The process for production of the cellulase-free xylanase including strain isolation has been covered under a US Patent (**Rele** et al. US Patent No. 5534429,1996**).** A cellulase-free xylanase from an alkali tolerant *Aspergillus fischeri* stable at a pH range of 5.0–9.5 has been identified by **Chandra Raj** and **Chandra** (*Biotechnol. Lett*. 17:309–14,1995).

D. Pectolytic Enzymes Pectolytic enzymes have been used for many years to reduce viscosity and improve clarity of fruit juices and vegetable juices and to increase their yields from their sources. Early attempts were made by growing moulds on moist bran heaps, which were dried and used directly as enzyme preparations. Presently solid state as well as submerged techniques under strictly aseptic and controlled fermentation conditions have ensured consistent and sustained enzyme productivities. *Aspergillus niger* and related species have been in commercial use for the manufacture of pectic enzymes. A complex of enzymes are involved in the degradation of pectin which include de-esterifying pectin methyl esterase followed by the chain

cleaving endopolygalacturonase. Pectin transeliminase which cleaves the glycosidic bonds in the esterified pectin also plays an important role in pectin degradation. **Tuttebello** and **Mill** (*Biochem. J.* 79:51,1961) studied a strain of *A. niger* in submerged culture and found that while synthetic media with pectin gave poor yields, complex media with organic nitrogen such as peanut meal gave high activities. Besides aspergilli, other fungi such as *Coniothyrium diplodiella* produced high levels of pectic enzymes in solid state fermentations using inexpensive raw materials such as rice bran, wheat bran and sugar beet pulp (**Miura** and **Endo**, US Patent 3,058,890,1962).

A complex mixture of pectinolytic and cellulolytic/hemicellulolytic enzymes referred to as Macerozyme has been marketed by Japanese manufacturers and find application in maceration of plant tissues in food industry applications as well as in basic studies in plant biotechnology in the preparation of plant protoplasts. The fungal sources for these enzymes have been strains of *Aspergillus niger* or mucoralean fungi such as *Rhizopus chinensis*. A cellulolytic enzyme of fungal origin termed Cellulase Onazuka, also of Japanese origin, is used in conjunction with the Macerozyme for protoplast isolation from diverse plant materials.

E. Proteolytic Enzymes Proteinases are the largest category of commercial enzymes both in tonnage and in value terms. Numerous uses are made of them and several different types of proteolytic enzymes are manufactured to meet diverse demands. Four major types of proteinases, which are endopeptidases, are distinguished. These are serine proteinases which have a serine residue at the active site, and which exhibit activity at a pH range of 8.0–11.0. The thiol proteinases use the –SH groups in catalysis and have a broad pH optima around neutrality. Carboxyl proteinases have acidic pH optima and use the carboxyl group, especially of aspartic acid in the catalytic mechanism. Metalloproteinases have bound metal atoms at the active sites which participate in catalysis and they are inhibited by chelating agents such as EDTA. They are normally most active in the neutral pH range.

Microbial sources for proteinases have been identified for commercial manufacture, especially the serine proteinases which find application in food, pharmaceutical and detergent industries, while carboxyl proteinase include rennin from calf rennet used in cheese making and pepsin from pig stomach.

Bacillus cultures, especially strains which are alkalophilic, have been identified and widely used for the commercial manufacture of alkaline proteases in very large quantities for applications in the detergent industry. There are only a few fungal proteinases with high alkali stability. One of the significant findings on fungal alkaline proteinase has been from the National Chemical Laboratory, Pune where strains of saprophytic entomophthoralean fungi belonging to the genus *Conidiobolus* were studied and shown to secrete high levels of alkaline proteases active at and stable to high pH values. The *Conidiobolus* enzyme showed compatibility with several commercial detergents and also was potentially useful in animal cell cultures as a substitute for conventional trypsin (**Srinivasan** et al. *Biotechnol. Lett*. 5:285–88,1983, **Phadatare** et al. *Enz. Microbial. Technol*. 15:72–76,1993, **Chiplunkar** et al. *Biotechnol. Lett*. 7:665–68,1985). The enzyme appears also to hold promise and potential for applications in leather biotechnology and also in the degumming of silk for improving the quality and properties of silk fabrics. Species of *Aspergillus* are the principal sources of acid proteases. In *A. oryzae*, the predominant enzyme is a carboxyl proteinase with pH optimum at 4.0–5.0. The other important acid protease with commercial potential is the milk clotting enzyme identified from fungi like *Endothia parasitica* ("Sure Curd") and species of thermophilic Mucorales like *Rhizomucor miehei* and *R. pusillus* (referred to earlier as *Mucor miehei* and *M. pusillus*). The enzyme preparations have a high ratio of milk clotting to general proteolytic activity and have been tried out as substitutes for calf rennet in cheese making. The milk clotting activity is due to its selective attack on the *k*-casein fraction, which stabilises the casein micelle in milk. *R. pusillus* enzyme was stable from pH 3.0–6.0 and showed maximum activity at 55 °C. Other sources of thermophilic fungi secreting acid proteases include

Penicillium duponti, which secreted the enzyme in submerged fermentation in rice bran media at 50 °C. (**Hashimoto** et al. *Appl. Microbiol.* 24:986–92,1972). Thermomycolase, an alkaline protease secreted by the thermophilic fungus, *Malbranchea pulchella* var. *sulfurea* (**Ong** and **Goucher**. *Can. J. Microbiol*. 22:165–76,1976) is a serine protease formed in presence of casein and was repressed by the presence of glucose, peptides, amino acids or yeast extract. Thermomycolase was active optimally at a pH of 8.5 and was stable over a pH range of 6.0 to 9.5 for over 20 hours at 30 °C. Calcium ions stabilised the enzyme at high temperatures.

Molecular Approaches to the Study of Industrially Useful Enzymes

Molecular approaches to the study of extracellular enzyme secretion are aimed at getting a better insight into the various molecular aspects such as zymogen processing, folding of the protein in the heterologous host system, effect of glycosylation on the enzyme secretion etc. The *Rhizomucor miehei* protease has been subjected to molecular studies and the recombinant protein expressed in *Aspergillus nidulans* having similar properties (**Gray** et al. *Gene* 41:53–53,1986). Using the alpha amylase promoter the enzyme protein has also been successfully expressed in *Aspergillus oryzae* with significant yields (**Christensen** et al. *Biotechnology* 6:1419–22,1988). A lipase gene from *Humicola lanuginosa* (*Thermomyces lanuginosus*) has been cloned and expressed in *Aspergillus oryzae*. Similarly *Rhizomucor miehei* lipase is also processed and expressed in transformed *A. oryzae* (**Huge-Jensen** et al. *Lipids* 24:781–85,1989). *H. lanuginosa* lipase is marketed by Novo industri under the trade name Lipolase as a detergent additive along with other hydrolases such as protease, amylase and cellulase. In a patent granted to **Novo Nordisk**, Denmark (W09630–502) (*Biotechnol. Abst*. 96–15062,1996), an alkaline lipase for detergent use from *Botryosphaeria* or *Guignardia* sp. and recombinant enzyme production in *Aspergillus* has been claimed. A method for producing recombinant alkaline lipase by isolating an alkaline lipase encoding DNA sequence from either fungus, combining the

sequence with expression elements in a vector, subsequently transforming a host (e.g. *Aspergillus* sp.) and then recovering the alkaline lipase from the culture medium have been described.

The composition of the extracellular complex of cellulase enzymes secreted by *Trichoderma reesei* comprises 60–80% of cellobiohydrolase, 20–30% of endoglucanase and less than 1% of the total secreted protein is β-glucosidase. Genetic engineering methods have been employed for producing strains with novel cellulase profiles. **Harkki** et al. (*Enz. Microbial. Technol*. 13:227–33,1991) used a general expression vector pAMH11O containing the promoter and terminator sequences of the strongly expressed cellobiohydrolase I (cbh I) gene to overexpress a cDNA for the major endoglucanase (EG I). An in vitro modified cDNA of cbh I incapable of coding for active enzyme was used to inactivate the major cellobiohydrolase gene. Thus new strains producing elevated amounts of all the specific endoglucanases and/or lacking the major cellobiohydrolase cbh I were produced. The Finnish group have also successfully used the strong and highly inducible promoter of the gene encoding the major cellulase (cellobiohydrolase I) for the production of eukaryotic heterologous protein, chymosin in *Trichoderma* (**Uusitalo** et al. *J. Biotechnol*. 17:35–50,1991).

Hodgson (*Biotechnology* 12:789–90,1994), reviewing the changing bulk biocatalyst market, pointed out that recombinant DNA techniques have changed bulk enzyme production dramatically and that 50% or more of the industrial enzymes measured by value or mass are from organisms that have been genetically engineered. High level of confidentiality maintained by industrial firms about the manufacturing processes has resulted in lack of authentic information on the actual levels at which recombinant strains are currently in use for commercial manufacture. It can however be envisaged that this powerful technique along with those of protein engineering are and will continue to be judiciously employed in developing novel biocatalysts with augmented levels of desirable properties in industrial enzyme technologies.

CHAPTER 10

Fungal Biology and Biotechnology

Present Status and Future Perspectives

The foundations of mycological science were firmly laid by those biologists who collected fungi from the natural environment, investigated their morphological features and established a base for their classification, taxonomy and identification. Physiological and biochemical studies in vitro on pure cultures expanded the horizons of knowledge further paving the path for utilising fungal systems in technological applications. In the context of our present-day knowledge and understanding of fungi, we are in a position to appreciate the potential that they hold for biotechnological innovations and inventions. It follows that in order to sustain future progress, emphasis must be laid on updating the knowledge base continuously in the biological as well as technological aspects of mycology.

The advance of mycological science during the past fifty years and its various facets of development have been discussed by several eminent mycologists. **Chester Benjamin** in his presidential address to the American Mycological Society entitled 'The changing face of Mycology' (*Mycologia* 60:1–8,1968) pointed out that (a) mycology is a dynamic discipline (b) mycology plays a continuing significant role in biology and agriculture (c) some of the problems in mycology must be solved in concert with other disciplines and (d) mycologists have opportunity to expand their contribution to science and human welfare. He also emphasised the need for extensive mycological studies by stating as follows: "There

would seem to be a need for greatly accelerated activity in both field and laboratory for our mycota to be even reasonably well understood in the next 50–75 years."

Ralph Emerson in his presidential address to the British Mycological Society (*Trans. Brit. Mycol. Soc*. 60: 363–87,1973) entitled "Mycological relevance in the nineteen seventies" discussed about the growing tendency for questioning the relevance of basic mycological investigations if such studies are not directly capable of being linked with development of beneficial and useful technologies. He concluded that fundamental knowledge about fungi is a most vital and essential prerequisite to harness mycological science for the benefit of mankind. Referring to an editorial entitled "Educational obsolescence" in *Science* (170:1041,1970) by **Bentley Glass**, he emphasised that "we do need to train our students for both the 'timely' application and the 'timeless' fundamentals." In the thirty years that have elapsed since **Emerson's** paper was published, one can observe a steady decline in the focus on the 'timeless fundamentals" in many aspects of mycological science, which is particularly regrettable.

C.V. Subramanian (*Current Sci.* 63:167–72,1992) discussed fungal biology and biotechnology with particular reference to tropical fungi and stated that "the relevance of tropical mycology to biotechnology rests on mycodiversity and the enormous fungal power that mycodiversity implies... ." There is an urgent need for a fungal inventory of the tropics, for a combing of special microhabitats and ecological niches for mycogenomes, the maintenance in culture and conservation of these genomes... ."

C.P. Kurtzman (*Proc. Asian Mycological Symp*., Seoul, South Korea p. 1–6,1992) commenting on the impact of mycology on the needs of the twenty-first century, listed several important contributions which fungi can make towards meeting the enhanced demands of the new millennium. These include (a) enzymes for conversion of agricultural products and residues to high value chemicals and to fuel (b) sources of unique genes for application in

biotechnology (c) Biocontrol of plant and animal pests and pathogens and (d) abatement of environmental pollution. The role of mycologists would be (a) to provide novel germplasm through discovery and characterisation of new species (b) to improve diagnostic taxonomic methods for recognition of new species for technology and (c) to interact with biotechnologists to exploit the many unique properties of fungi.

Several contributions from mycologists have helped in achieving a better understanding of the basic physiological and biochemical aspects of life processes including morphogenesis and differentiation. The Nobel prize winning contributions of **Beadle** and **Tatum** on *Neurospora* genetics is an excellent example where observations on the life cycle of the fungus in vitro led to elucidation of the biochemical basis of inheritance in higher forms of life including the one gene–one enzyme hypothesis. Recent years have seen increased focus on fungal systems as sources of useful metabolites and enzymes including their application as heterologous hosts for expression and manufacture of mammalian proteins, the details of which have been discussed in the earlier chapters. What are the specific areas in which the knowledge base needs to be sustained and continuously strengthened? Three of the most important aspects which require emphasis are (a) a deeper understanding and appreciation of natural mycodiversity (b) a meaningful taxonomy which will enable ready recognition and differentiation of one taxon from the other and (c) physiological and biochemical data helpful in conservation of pure cultures in germplasm banks ensuring morphological and genetic stability.

Fungal biodiversity necessitates careful analysis of various natural substrates at various stages of decomposition and there is increased awareness of the potential offered for discovering hitherto undescribed species from such studies. Innovative approaches including the use of selective media formulations to enumerate the less common and slower growing species would expand the scope of these studies and also make them more meaningful for a better understanding of natural fungal biodiversity. An insight into the ecology and natural distribution along with taxonomic expertise of a high

order would make these studies even more fruitful. Unfortunately in the present times, the number of qualified personnel who have genuine interest in undertaking such exploratory studies is very low. 'The dwindling number of individual scientists with expertise in the isolation of specific types of microorganisms from nature poses a serious problem and in some cases this ability is becoming a lost art" (**Labeda**, in preface to *Isolation of Biotechnological Organisms from Nature*, McGraw-Hill Publishing Co., N.Y. 1990, 322P). Also the levels of expertise in taxonomy and classification based on morphological features and specialisation in the taxonomy of specific groups of fungi among researchers has rapidly dwindled and in many situations molecular taxonomy is being projected as the only basis for realistic classification. It should be realised that molecular data generated on fungal systems could add useful adjuncts in arriving at some meaningful conclusions with regard to clarifying taxonomic relationships. However, molecular taxonomy cannot be a replacement for morphology-based taxonomy and classification. As emphasised by **Subramanian** (*Current Sci*. 63:167–72,1992) there is an urgent need for mapping our enormous and rich fungal resources and we need an inventory of the fungal taxa, species, genera and families. A culture collection of tropical fungi needs to be built up as also a strengthening of knowledge of their genetics, physiology and biochemistry. In the case of genera with overlapping morphological features, it is possible that molecular data could be generated to differentiate specific industrial isolates and assign them to one or the other taxon. In mycological literature several genera and species have been described for which the pure cultures are not available for enabling further investigations. In other cases, the genera are monotypic and if at all, the cultures may be available only in the reputed culture collections abroad and paradoxically many of these would have been isolated from tropical locations of the developing world. It is now well established that the tropical regions hold the maximum fungal diversity and mycologists working in the laboratories located in tropical countries should make serious attempts to identify and isolate in pure cultures more representatives of the rare or monotypic genera and build up specialist germplasm banks with sufficient number of

isolates. These then could be taken up for molecular biological investigations as well as biotechnological explorations. Specialisation and strengthening of the knowledge base among individual mycologists would lead to the establishment of active research schools of excellence, which could function in future as reference centres with authoritative data bases for specific groups of fungi. In the context of biotechnology patenting and protection of intellectual property rights, fungal taxonomy has acquired a new importance and significance. The proper nomenclature and identification of the production strain is essential while filing patent applications and an improper designation of the strain could lead to rejection of the patent claims.

Biotechnology processes are based on strains modified through induced mutations and/or recombinant DNA technology. Such strains selected for hyperproduction of the desired metabolite may not strictly conform to the morphological and sporulation pattern(s) of the original wild strain on which the identification is based. Molecular studies could possibly address to the meaningful identification of features, which could be regarded as authentic or diagnostic for specific strain identification. Presently serious efforts in this direction appear to be very limited and it would be obvious that classical mycologists and molecular biologists would need to pool up their expertise together in order to generate newer information and knowledge bases, which are applicable for such diagnostic purposes.

Physiological and biochemical knowledge pertaining to in vitro behaviour during prolonged cultivation in pure cultures is yet another important area calling for serious attention from mycologists and biotechnologists. There is no universal foolproof method available ensuring morphological and genetic stability of all fungal cultures during prolonged maintenance in artificial culture on nutrient media. The diversity of response to different conservation techniques needs to be carefully analysed before standardising the protocols ensuring sustained viability as well as genetic stability. Future years will see the increased use of fungal strains carrying heterologous genes, especially for proteins of

mammalian origin and special techniques to ensure stability of the cloned genes, perhaps through conservation techniques like cryopreservation applied to the mycelial state of the strains, may require optimisation and standardisation on individual strain basis.

A discussion on how culture collections are important for biotechnology research and development and the type of expertise and organisation would be essential for practical biotechnology would be worth considering. Three types of culture collections can be identified: (a) service collections with the objective of providing authentic cultures for biotechnological research and development and be concerned with standardisation and implementation of long-term conservation strategies, especially for sensitive or fastidious cultures as well as genetically engineered strains. These should also act as International Depository Authority (IDA) for patent strains. As service utilities, these culture collections need to be ensured continued and long-term support with substantial grants and adequate infrastructural support from public/government funding towards facilities, employment of technical staff with high level professional skills in microbiology and molecular biology; (b) in-house collections which are built up by commercial organisations based on their specific and often specialised biotechnological interests and comprise strains accessioned from other collections and modified to meet their specific demands as well as strains isolated from natural resources. Obviously information on the details of the cultures maintained or investigated by commercial organisations would not be made public and very little scientific information based on their studies would be available through publications in scientific journals; (c) research collections based on the research interest of individual scientists or scientific groups which are often based on high-level expertise of the personnel and these studies would be invaluable for the data related to strains investigated. Data published in scientific journals would be available for reference but often the strains would be lost after the particular project work is completed since the research interests of the scientists may shift to other areas. In the interest of conserving these valuable germplasm for future investi-

gations, the strains must be deposited in one or more of service collections and these service collections should access and conserve them taking all the necessary care for their sustained and long-term maintenance without undergoing pleomorphic degenerations.

Natural product-based drug discovery and the screening for bioactive molecules from plants, animals and microorganisms is a major activity in leading biotechnology laboratories around the world and is presently regarded as competitive with high throughput screening and combinatorial chemistry and genomics. Actinomycetes and fungi are among the most preferred and according to a recent report 73% of Japanese commercial establishments were actively conducting such screening based on fungal systems for new molecules (**Okuda**. *WFCC Newslett*. 35:21–26, July 2002). It is firmly believed that these programmes would give lead structures 'beyond the imagination of the synthetic chemist'. In-house taxonomic expertise has been recognised as indispensable for undertaking such studies and this report makes special mention of the Japanese companies deputing their technical staff for 'on-the--job' training programmes in mycological taxonomy in collaboration with the Mycological Society of Japan. Ascomycetes and mitosporic fungi were screened from soil or plant litter while less emphasis was given to Oomycetes and Zygomycetes from which not many secondary metabolites of value have emerged.

There are many challenging areas in basic mycology, which require thorough investigation for a better understanding. For example, many fungi, which are pathogenic on plants, have not yet been successfully cultured under artificial conditions and maintained in vitro and the term obligate pathogens has been conventionally applied to them. Likewise a group of saprobic Ascomycetes associated with insects, the Laboulbeniales on which **Roland Thaxter** made such outstanding pioneering contributions, have neither been extensively investigated nor cultured on laboratory media. The rust fungi which are among the most serious pathogens of various crop plants including cereals like wheat have so far defied routine culturing. However some reports of successful culturing of the wheat rust fungus *Puccinia graminis* (**Scott** and **Maclean**.

Ann. Rev. Phytopathol. 7:123–46,1969, **Foudin** and **Wynn**. *Phytopathology* 62: 1032–40,1972) clearly seem to warrant the necessity for more intensive investigations, not only on various rust fungi but also on the other so-called obligate pathogens which include, for example, the downy mildews (*Sclerospora*, *Sclerophthora* and *Plasmopara*), the powdery mildews (*Erysiphe*, *Oidium*, etc.), the aquatic mosquito larval pathogen *Coelomomyces* and the endophytic pathogens of protozoa classified under the Zoopagales. The identification of specific growth factor requirements to enable their culture on laboratory nutrient media in the absence of the living host is scientifically challenging and if success is achieved, the biodiversity of the presently uncultured fungal taxa could add a new dimension for biotechnology exploration. If the scientific basis for their culture and in vitro conservation were established, their biodiversity would add a new dimension for exploring them.

The term 'mycotechnology' was coined by **Bennett** (*J. Biotechnol*. 66: 101–107,1998) to "describe the enormous impact of fungi on biotechnology". The term is designed "to include the many biotechnological processes both old and new that rely on fungal products and processes. These lower eukaryotes remain important models for basic biology and commercial manufacture. In discussing "post modern mycotechnology" spawned by "marrying the techniques of gene splicing and other post-recombinant DNA methodologies to those of conventional industrial mycology", **Bennett** has listed the following as the most significant: (a) expression of heterologous gene of fungal, plant or mammalian origin; (b) amplification of homologous genes with particular reference to pathways of antibiotic synthesis, industrial enzyme production; (c) manipulation of secondary metabolite pathways leading to the development of new semi-synthetic as well as hybrid antibiotics; (d) more intensive analysis of fungal genomics and genetic transformation systems, with a view to accelerate drug discovery process using DNA chips in conjunction with combinatorial chemistry and assay miniaturisation. These modern techniques, especially DNA chips can be used to identify important molecular targets for drug discovery.

To illustrate the approaches combining classical and molecular techniques we may discuss two examples with reference to (a) development of newer antifungal agents and (b) enzyme technology. As discussed in an earlier chapter, opportunistic fungal infections of immuno-compromised patients is a serious development. It is important to develop new broad-spectrum antifungals which are fungicidal rather than fungistatic. Also the identification of molecular targets would lead to newer approaches in antifungal therapy. The newer targets preferably need to show wide distribution within the fungal kingdom in order that broad-spectrum, target-directed antifungals can be identified from natural as well as synthetic compounds by employing high throughput screens. Potential selective antifungal targets are beginning to emerge from studies on proteins that modulate DNA topology, gene transcription, m-RNA translation and protein modification. The translation elongation factor EF-3 is one component of fungal protein synthesis machinery that would be a potential target. EF-3 is a soluble factor which interacts with fungal ribosomes to release uncharged t-RNAs and it is considered that inhibiting either its ribosome-dependent ATP-ase activity or its ability to bind to the ribosome should provide an effective antifungal strategy. EF-3 has been over expressed in engineered *Saccharomyces cerevisiae* and a compound RG-530 has been identified which is inhibitory to *Candida albicans* in culture at levels below 1 μg/ml (**Tuite**. *Trends Biotechnol*. 14:219–20 ,1996).

In the area of enzyme technology, detergent, food and starch processing industries account for more than 75% of industrial enzyme usage involving hydrolases such as proteases, amylases, lipases and cellulases. Recent reports indicate that over 60% of such enzymes in use are recombinant products including those, which have been modified by site-directed or random mutagenesis to enhance one or more functional properties. For example, individual components of the *Trichoderma reesei* cellulase complex (endoglucanase, exoglucanase, and β-glucosidase) have been intensively investigated leading to an improved understanding of cellulase activity relative to molecular structures of different

cellulose polymers. This knowledge has in turn enabled development of customised cellulases for specific 'stone washing' processes used in the textile industry (**Cowan**. *Trends in Biotechnol*. 14:177–78,1996).

Exploration of the potential of fungi for other diverse applications has also gained considerable momentum in recent times. These include their use as biocontrol agents in the control of plant diseases, biopesticides and as mycoherbicides for weed control. The use of selected mycoparasites to control soil borne plant pathogens was reviewed by **Adams** (*Ann. Rev. Phytopathol*. 28:59–72,1990). These include *Trichoderma* species such as *T. harzianum* effectively reducing disease incidence by *Sclerotium rolfsii* and *Rhizoctonia solani* and *Talaromyces flavus* and *Coniothyrium minitans* acting as mycoparasites of *Rhizoctonia solani* and *Sclerotinia* species such as *S. sclerotiorum*. Biocontrol of insect pests by mitosporic fungi like *Beauveria bassiana*, *Metarrhizium anisopliae* and *Nomuraea rileyi* is a widely studied subject in the present context of exploring eco-friendly alternatives to toxic chemical pesticides. Extensive literature on techniques of mass sporulation of these fungi and their application for effective biocontrol of targeted insect pests is available. **Templeton** et al. (*Ann. Rev. Phytopathol*. 17:301–10,1979) have reviewed biological weed control with mycoherbicides. Fungal pathogens of plants applied as sprays that uniformly kill or suppress weed growth are termed mycoherbicides. An example of such a successful application is the control of weeds like *Aeschynomene* and *Jussieua* in rice fields by application of host specific *Collectotrichum gloeosporioides*.

In the area of pollution control and waste treatment several fungi have been studied for biosorption of heavy metals, decolorisation of dye effluents and degradation of toxic residues. In the paper industry basidiomycetous fungi like *Trametes versicolor* have been found to be promising in treating the effluents successfully. In recent years fungi and yeasts have been successfully employed in the synthesis of semiconductor nanoparticles for use in fabrication of diodes. **Kowshik** et al. (*Biotechnol. Bioeng*. 78: 583–87, 2002) reported intracellular synthesis of cadmium sulphide

nanoparticles by a strain of *Schizosaccharomyces pombe* when challenged with 1 mM cadmium in solution. Likewise a strain of *Torulopsis* synthesised intracellular nanocrystallites of lead sulphide that exhibited semiconductor properties (**Kowshik** et al. *Adv. Mater*. 14: 815–18, 2002). Species of *Verticillium* and *Fusarium oxysporum* have been identified which when exposed to aqueous gold, silver and cadmium salt solutions showed intracellular/extracellular reduction of the metal ions and the nanoparticles exhibited tolerable monodispersity and in the case of particles synthesised extracellularly excellent long-term stability was observed. (**Ahmad** et al. *J. Amer. Chem. Soc*. 124:12108–9.2002, **Ahmad** et al. *Proc. 29th Ann. Meet. of Mycol. Soc. of India*, Pune Feb 2003 (Abstracts)).

Biotechnology is a highly competitive, multidisciplinary science. With the emerging trends towards globalisation of science and technology, intellectual property rights (IPR) protection and a clear understanding of International Patent Laws is of utmost importance. More important perhaps is the necessity for intellectual property generation, which needs to be adequately protected through international patenting in order to enable us to stay competent and competitive in the global scenario. Developing countries like India with high levels of intellectual competence must start placing emphasis on intellectual property generation which will be accepted as new, original, innovative and competitive. If properly explored in well-organised screening programmes, natural fungal biodiversity from the Indian subcontinent may be expected to make meaningful contributions to the generation of new and novel technologies which would be globally competitive. The updating and revision of the Indian patent laws, the signing of the Budapest treaty regarding deposition of the patent strains and most recently, the recognition granted to an Indian culture collection (Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh) as an International Depository Authority (IDA) are all positive steps in the right direction to provide the proper climate to go about with intellectual property generation and its protection. In order to play a pivotal and central role in such activities, mycologists must prepare themselves for a different mind-set. While continuing to excel

in their chosen fields of taxonomy and cataloguing of mycoflora including description of new species, they must also study the fungi in pure culture and optimise the protocols for effective long term in vitro conservation. They should attempt to build up specialist germplasm collections of their fungal isolates in-house and also deposit their strains in the national germplasm banks with full details of their scientific findings on the fungal strains studied by them. This will ensure that a national 'mycological heritage' will be developed and conserved for mycological and biotechnological investigations in future years. Mycologists should also recognise and appreciate their role as specialists contributing to progress in the integrated science of biotechnology in collaboration with biochemists, organic chemists and bioprocess engineers. Mycological aspects of process development including aspects related to morphogenesis, differentiation and metabolite overproduction under fermentation conditions can only be optimised if studied from a multidisciplinary approach involving high-level expertise in different scientific disciplines.

APPENDIX 1

Glossary

General Mycological/Microbiological Terms

Arbuscule: A finely branched structure produced by endomycorrhizal fungi within the root cells and at its interface exchange of phosphorous and photosynthates take place.

Autotrophic: Organisms capable of synthesis of energy-rich carbon compounds using energy from light or inorganic reactions and not using organic compounds as primary sources of energy (see Heterotrophic).

Auxotroph: Biochemical mutant deficient in synthesis of specific growth substances, whose supplementing to minimal medium is essential to support growth.

Axenic culture: Condition in which a culture grows independent of other living systems present such as host, symbiont or parasites.

Binomial: Name given to each known species composed of a generic and specific epithets (e.g. *Aspergillus niger*).

Chemotaxis: Response (e.g. motility of free swimming cells like zoospores) to chemical stimulus.

Clamp connection: Short backwardly directed hyphal growth of dikaryotic Basidiomycetes, which at the time of cell division establish connection between adjacent daughter cells.

Cloning: Transfer of select genes from a donor host cell into a recipient system through vectors (e.g. plasmids) and culturing the "transformed" recipient carrying the alien gene.

Coenocytic: Condition in which the hyphae are multinucleate and lacking cross walls (e.g. Oomycota, Zygomycota).

Crustose: Closely adhering to the surface (e.g. Crustose lichens adhering to rocks).

Dikaryon: A nuclear phenomenon seen in Ascomycetes and Basidiomycetes wherein the compatible nuclei pair and divide synchronously without undergoing fusion.

Dimorphic: Condition of having two distinct forms (e.g. yeast-mycelial dimorphism) exhibited by pathogenic fungi like *Histoplasma capsulatum*. The yeast phase is seen in human infection mycelial state in axenic cultures. Yeast phase of some mucoralean fungi induced experimentally and show fermentative metabolism.

Diploid: Having two compliments ($2n$) of haploid chromosomes in the nucleus (e.g. Zygote nucleus after nuclear fusion in gametes).

Dolipore: A unique barrel-shaped septal pore present in dikaryotic Basidiomycetes hyphae and formed by central dilation. It is usually observed through electron microscope.

Eucarpic: A thallus, which is differentiated into vegetative and reproductive structures (see Holocarpic).

Eukaryotic: Having nucleus with a well-defined nuclear membrane, specific number of discrete chromosomes and dividing mitotically (see Prokaryotic).

Evanescent: Disappearing rapidly, having a short existence.

Flagella (Sing. Flagellum): Whip-like locomotary organelle present in motile cells (e.g. Zoospores). These are tubular extensions which are bounded by plasma membrane (see Whiplash and Tinsel Flagella).

Germ pore: A thin area of spore wall through which the germ tube emerges upon germination.

Germ tube: The emerging young hypha from a germinating spore.

Gene: Unit of heredity in the chromosome, sequence of nucleotides in DNA molecule responsible for coding of specific polypeptide/protein.

Genome: The total genetic component of an organism comprising genes in the nucleus as well as cytoplasm (in case of Eukaryotes).

Genotype: The sum total of an organism's genetic potential of which only a part (phenotype) is expressed at any one time.

Haploid: Having a single set of chromosomes (designated as *n*) (e.g. gametes carry haploid number of chromosomes in all biological systems) (see Diploid).

Hartig nest: The mycelial web formed by an ectomycorrhizal fungus in association with the surface layer of host root system for effective symbiosis (see Mycorrhiza).

Haustorium: A specialised absorptive structure by which parasitic fungi penetrate the host cell and absorb nutrients for growth and sustenance.

Heterokaryotic: Mycelia containing nuclei which are genetically dissimilar.

Heterotrophic: Incapable of deriving energy from photosynthesis or inorganic chemical reactions but using organic compound as energy source (see Autotrophic).

Heterothallic: Fungi in which two genetically distinct but compatible mycelia must come together for sexual reproduction to take place (see Homothallic).

Holocarpic: Thallus getting converted entirely into reproductive structure (see Eucarpic).

Homothallic: Fungi which are self-compatible and lacking in different mating types. Sexual reproduction can take place in mycelial colonies originating from a single spore (see Heterothallic).

Intercalary: Occurring or developing between cells (e.g. intercalary chalmydospores in *Fusarium*).

Lyophilisation: Freeze-drying technique widely applied for conserving fungi in collection with minimal genetic variation over prolonged periods.

Mastigonemes: Hair-like processes on the surface of 'tinsel' flagella.

Meiosis: Reduction division process by which diploid nucleus through two successive divisions undergoes reduction from $2n$ state to haploid (n) state together with genetic segregation and recombination (see Mitosis).

Merosporangium: Cylindrical finger-like outgrowths borne on swollen vesicles terminating sporophores and developing spores by internal segmentation (e.g. *Syncephalastrum* and other merosporangiferous Mucorales, Zygomycetes).

Mitosis: Nuclear division in which replication and equal distribution of chromosomes take place between two daughter cells, characteristic of eukaryotic vegetative cell division (see Meiosis).

Monilioid / Moniliform: Bead-like with regular swellings at intervals (e.g. microconidial chains of *Fusarium moniliforme*).

Monokaryon: Haploid phase in Ascomycetes and Basidiomycetes in which the hyphae contain only one kind of nucleus (see Dikaryon).

Muscardine disease: General term to denote fungal diseases of insects caused by hyphomycetous fungi (e.g. Green Muscardine disease by *Metarrhizium anisopliae,* white muscardine disease by *Beauveria bassiana* and yellow muscardine disease by *Paecilomyces farinosus*).

Mushroom: Fleshly basidiomycetous fruit bodies often with a stalk supporting a cap (pileus) in which the lower surface is composed of gills differentiating the basidiospores in well-organised hymenia. These include poisonous (*Amanita*) or edible (*Agaricus*, *Volvariella*) species.

Mutagen: An agent, physical or chemical, which can bring about permanent, heritable changes in the genetic composition of biological systems.

Mutant: An organism carrying mutated gene(s) often with altered physiological, morphological or biochemical characteristics.

Mutation: Permanent heritable changes affected in the composition of a gene.

Mycelium (Plural: Mycelia): Collection of vegetative hyphae in a growing thallus of a fungus.

Mycobiont: The fungal component in a symbiotic association (e.g. Lichens, Mycorrhizae) (see Phycobiont).

Myco-herbicide: Phytopathogenic fungi formulated to kill weeds.

Myco-insecticide: Insect pathogenic fungi formulated to control and kill insects.

Myco-parasite: Fungi attacking and parasitic on other fungi (e.g. *Piptocephalis* on Mucoraceous fungi, *Darluca* parasitic on rust sori).

Mycorrhiza: Symbiotic association between roots of higher plants and specific filamentous fungi. The association can be of two types as listed below:

(a) **Ectomycorrhiza** colonise outer zone of root surface and grow between cells of the root cortex (see Hartig Nest).

(b) **Endomycorrhiza** or vesicular arbuscular mycorrhiza (VAM) are endophytic fungi associated with plant roots and forming vesicles, classified under the order Glomales.

Mycoses: Fungal diseases of animals and humans (e.g. Ringworm, Athlete's foot, Histoplasmosis, etc).

Parasexuality: Genetic recombination during mitotic cycle of conidial fungi such as *Aspergillus*, Somatic recombination in the absence of a true sexual cycle.

Phenotype: The state of physical expression of the genotype.

Phycobiont: The algal (photosynthetic) partner in the symbiotic association with fungi (in lichens).

Phylogeny: Evolutionary history and mutual relationships among a group of organisms.

Pileus: The head or spore bearing "cap" in mushrooms.

Plasmid: Small circular DNA fragments with autonomous replication in the cytoplasm which can be used as a vector in gene cloning.

Prokaryotes: Organisms lacking a nuclear membrane or differentiation of the genetic material into chromosomes, also lacking cytoplasmic organelles like ribosomes, mitochondria and golgi apparatus (e.g. Bacteria, Actinomycetes and Cyanobacteria).

Promoter: A nucleotide sequence in a gene involved in the transcription of mRNA by attachment to RNA polymerase.

Protoplast: Cellular contents of a membrane-bound structure from which cell wall has been removed by enzyme treatment.

Psychrophilic (Cryophilic): Organisms growing optimally at temperatures below 10°C and showing poor growth above 20°C.

Rhizoid: Branched, enucleate thin assimilative filaments found associated with unicellular Chytridiomycota.

Rhizomorph: Root-like aggregation of hyphae with a well defined apical growing zone and frequently differentiated into a central core of elongated colourless cells and a peripheral rind

of small dark coloured cells, characteristic of Basidiomycetes like *Armillaria*.

Rhizomycelium: A rhizoidal system which appears similar to true mycelium, associated with some chytrids like *Cladochytrium*.

Ringworm: Superficial mycoses in humans and animals caused by keratinophilic Hyphomycetes such as *Trichophyton* and *Microsporum*.

Saprobe: Heterotrophic organisms depending for their nutrients on material derived from dead organisms or organic nutrients synthesised by other living systems.

Sclerotium: Firm frequently rounded hyphal mass, sometimes associated with host tissue and normally without any spores (e.g. *Sclerotium rolfsii*, *Sclerotinia sclerotiorum*).

Septa: Cross wall partitioning fungal mycelia into multiple segments often with pores permitting continuity of cytoplasm and nuclei among different segments. Transverse and/or longitudinal septa formation seen in many hyphomycetous spores.

Somatic: Refers to vegetative or assismilative body of an organism.

Soredia: Fragments of lichen thalli comprising fungal hyphal aggregates enclosing algal cells, acting as propagules.

Squamulose: Consisting of small scales (lichen thalli).

Sterilisation: Process of eliminating all micro-organisms and their propagules from a material by killing through exposure to heat (autoclaving), radiation, chemicals (e.g. ethylene oxide) or removal through suitable membrane filtration.

Subiculum: A wool-like or crusty growth of mycelium under fruit bodies.

Teratogenic: Causing abnormalities in growth and differentiation of embryonic tissues (e.g. in developing foetus).

Thallus: The vegetative body of a thallophyte, popular reference to a growing fungus colony.

Thermophilic: Thermophilic fungi grow optimally above 40–50°C while thermotolerant species grow optimally at 30–35°C and are capable of growth up to 45ºC (e.g. *Apergillus fumigatus*).

Tinsel flagellum: Flagellum with lateral flimmers or mastigonemes.

Transformation: A genetic change brought about by the introduction of foreign DNA.

Volva: A sheath surrounding the stipe of some mushrooms and appearing like a veil (e.g. *Amanita*).

Water activity (*a*

Ecology and Biodiversity

Aero-aquatic fungi: *Fungi inhabiting* ponds and streams with floating propagules, which colonise, leaves shed into the aquatic habitat. Spores have radiating arms or appendages aiding buoyancy.

Aerobiology: Study of fungal spores (and other propagules such as pollen) in relation to their occurrence and distribution in the atmosphere.

Bracket fungi: Persistent fruit bodies of Basidiomycetes such as the polypores appearing as brackets colonising dead wood.

Brown rot fungi: Fungi belonging to Basidiomycetes causing degradation of wood cellulose but not attacking lignin (see White rot fungi).

Coprophilous fungi: Dung inhabiting fungi, primarily belonging to Zygomycetes (e.g. *Pilobolus*), Ascomycetes (e.g. *Chaetomium*, *Sordaria*) and Basidiomycetes (e.g. *Coprinus*).

Coral fungi: Hymenomycetes (Basidiomycetes) with branched fruit bodies (basidiomata) (e.g.Clavariaceae, Aphyllophorales).

Dermatophytes: Fungi causing skin diseases on man and animals with keratin utilising capability (e.g. *Trichophyton*, *Microsporum*).

Downy mildew: Fungi belonging to Oomycetes (Peronosporales) causing serious infections on plants, Not cultured in axenic culture (*Sclerospora* on cereals and grasses, *Plasmopora* on grapes) (see Powdery Mildew).

Endophytic fungi: Fungi present in the tissues of living plants in a symbiotic existence without causing infection.

Entomogenous fungi: Fungi living in or on insects, often causing pathogenesis.

Keratinophilic fungi: Fungi capable of attacking and degrading keratin many of which cause superficial mycoses (see Dermatophytes).

Opportunistic fungi: Fungi, which are normally saprophytic causing infection and damage to host under some conditions such as the *Aspergillus* infection observed in immunocompromised human patients.

Osmotolerant fungi: Fungi capable of growing under conditions of high osmotic pressure, spoiling high sugar content substances like fruit juices.

Polypores: Shelf or bracket fungi forming perennial woody fruit bodies in which the hymenium lines layers of vertically oriented tubes.

Powdery mildew: Plant diseases caused by obligately pathogenic ascomycetous fungi (Erysiphaceae) (see Downy mildew).

Predaceous fungi: Diverse fungi attacking and killing protozoa and nematodes (e.g. Zoopagales, Hyphomycetes).

Rusts: Plant diseases caused by basidiomycetous obligate pathogens (Uredinales) often with a complex life-cycle involving alternate hosts (Heteroecism).

Shii-Take: Edible basidiomycetous fungus (*Lentinus edodes*) widely cultivated on fallen oak logs in China and Japan.

Smuts: Basidiomycetous fungi (Ustilaginales) pathogenic to plants and causing serious diseases on cereal crops like wheat and sorghum replacing the grain with powdery mass of black spores.

Stinkhorn: Fruit bodies of basidiomycetous fungi producing strong stench attractive to carrion flies, which aid in spore dispersal (e.g. Phalloids).

Trichomycetes: A group of Zygomycetes having specific association with hindguts of millipedes, centipedes, etc.

Truffle: Edible ascomata of the genus *Tuber* (Pezizales, Ascomycetes).

Water moulds: Aquatic fungi belonging to Saprolegniales (Oomycota).

White rot fungi: Basidiomycetous fungi degrading both cellulose and lignin (e.g. *Pleurotus*, *Phanerochaete*) (see Brown rot fungi).

Classification and Taxonomy

Ascomycetes: Large group of saprophytic/pathogenic fungi with formation of ascopores within sac-like structures termed ascus. The ascopores are products of sexual recombination and the asexual (conidial) state of many Ascomycetes belong to the Hyphomycetes.

Basidiomycetes: Large group of saprophytic or phytopathogenic fungi in which the basidiospores, which are products of genetic recombination are borne on basidia (usually four per basidium) on sterigmata (e.g. mushrooms, rusts, smuts).

Chromista: A composite group of unicellular or multicellular forms considered to be related to and derived from photosynthetic algal ancestors. Three orders Hyphochytriomycota, Labyrinthulomycota and Oomycota are recognised.

Coelomycetes: Conidial fungi in which spores are produced within a protective or enclosed structure (e.g. acervulus, pycnidium).

Cup fungi: Ascomycetes with open cupulate apothecia containing the asci and ascopores (Discomycetes, Pezizales).

Ear fungus: Auriculariales (Basidiomycetes) with gelatinous edible basidiomata.

Earth balls: Nonostiolate fruit bodies of Sclerodermataceae (Basidiomycetes).

Earth stars: Star-shaped fruit bodies of *Geastrum* (Basidiomycetes).

Ergot/ergotism: Disease of cereals and grasses in which horny sclerotia (Ergot) replace normal grain development (*Claviceps purpurea*) on rye. Ergotism is ergot poisoning in man and animals leading to gangrene, convulsions (also termed "St. Anthony's fire"), etc.

Eumycota: Term which refers collectively to "True fungi" comprising the Chytridiomycota, Zygomycota, Ascomycota and Basidiomycota (see Oomycota).

Family: Taxonomic grouping composed of genera and ranked below "order", indicated by the suffix—aceae (e.g. Saprolegniaceae).

Fungus: Non-photosynthetic heterotrophic eukaryotes absorbing nutrients from organic substrates through enzymatic degradation, uni- or multi-cellular, classified under Chromista or Eumycota.

Fungi imperfecti: An unnatural assemblage of fungi known only from conidial state (anamorph). Use of this term is no longer preferred (see Mitosporic fungi).

Hyphochytriomycota: Phylum classified under Chromista. Zoospores anteriorly uniflagellate.

Kingdom: Refers to the highest taxonomic category of life forms of which seven are recognised, viz. Archaebacteria, Eubacteria, Chromista, Protozoa, Eumycota, Plantae and Animalia. Fungi are grouped under Eumycota and two phyla under Chromista.

Labyrinthulomycota: Classified under Chromista and include the Labyrinthids and Thraustochytrids. Marine and fresh water forms associated with plants and algal chromists, saprobic, some are pathogens.

Lichens: Symbiotic association between fungus (Ascomycetes or Basidiomycete) with photosynthetic green alga or cyanobacteria.

Mitosporic fungi: Term applied to all fungi known only in the anamorphic (conidial) state and whose perfect (teleomorphic) state is not present or not yet discovered. Spores contain nuclei resulting from mitotic division (see Fungi Imperfecti).

Morel: Edible ascomycetous fruit body of *Morchella* (Pezizales).

Nomenclature: Naming of fungi in accordance with the guidelines prescribed by International Code of Botanical Nomenclature and binomial nomenclature of genus and species.

Oomycota: Classified under Chromista, Forms with biflagellate zoospores, cellulose walls and diploid vegetative thalli, regarded as related to photosynthetic algae, saprobic water

moulds (Saprolegniaceae) and plant pathogens (*Pythium*, *Phytophthora*).

Order: Taxonomic rank above family, suffix–ales (e.g. Mucorales).

Phylum: Term for grouping in each kingdom. Each phylum is further classified into class, order, family, genera and species.

Protozoa: Unicellular,plasmoidal or colonial eukaryotes, phagotrophic (food ingesting), consist of four phyla related to fungi via. Acrasomycota Dictyosteliomycota, Myxomycota and Plamodiophoromycota.

Species: Lowest rank in classification of an individual, technically designated under genus (generic and specific epithets occur together (e.g. *Aspergillus niger*).

Taxon (plural Taxa): Grouping of organisms for purpose of systematic classification ranging from species to kingdom.

Zygomycota: Phylum of True Fungi (Eumycota) with asexual reproduction through one to many-spored sporangia, and sexual reproduction by mating gametangia—differentiated in vegetative mycelia lacking motile spores—with flagella, Mucorales and Entomophthorales are the most important orders under Zygomycota. Zoopagales, which are parasitic on amoebae, are also classified under this phylum.

Sporulation / Spore Forms

Acropetal: Description of conidial chains in which the youngest conidium develops at the tip of the chain (see Basipetal).

Anamorph: Asexual reproductive state of a fungus, especially conidial state of mitosporic fungi (see Teleomorph).

Anisogamy: Gametes or gametangia which undergo fusion are of different sizes (Oomycota, Zygomycota).

Annellidic: Conidial production in sequence by short extensions of conidiophores which leave annular scars.

Antheridium: Male gametangium, well developed in Oomycota (see Oogonium).

Apothecium: Saucer or cup-shaped ascomata in which the hymenium is exposed at maturity (e.g. Discomycetes).

Ascogenous hyphae: Dikaryotic hypha growing out of the ascogonium after fertilisation, eventually differentiating the asci.

Ascogonium: Female gametangium in Ascomycetes.

Ascus/ Ascospores: Sac-like distinguishing structure in Ascomycetes bearing usually eight (in some cases numerous) ascospores.

Asexual reproduction: Form of reproduction not involving meiosis but only mitotic nuclear division (see Sexual reproduction).

Azygospore: Zygospore-like structures formed parthenogenetically without true gametangial copulation in some Mucorales.

Ballistospores: Spores discharged with force from its point of origin (e.g. basidiospores of mushrooms).

Basidium: The structure in Basidiomycetes which develop pointed sterigmata bearing basidiospores (usually 4 per basidium).

Basipetal: Conidial chain in which the young conidia are differentiated at the base of the conidiophore (see Acropetal).

Buller's drop: Tiny liquid droplet which develops at the base of the basidiospore prior to its active discharge.

Chlamydospore: Asexual spore, generally thick walled and formed by localisation of cellular contents and serving for perennation rather than dissemination of the species.

Cleistothecium: An ascomata which is completely closed in which the evanescent rounded asci are not arranged in a regular hymenium, characteristic of Eurotiales.

Columella: Extension of sporophore into the spore bearing structure (e.g. in the sporangia of *Mucor*).

Conidiation: Process of producing conidia.

Conidiophore: Specialised branch of vegetative mycelium, simple or branched on which conidia are differentiated.

Conidium (plural Conidia): Non motile fungal mitospore formed on characteristic conidiophores and not formed in sporangia.

Conjugation: Sexual fusion between somatic cells differentiated into gametangia in Zygomycetes.

Crozier: Terminal hook in ascogenous hyphae in Ascomycetes in which conjugate nuclear division occurs before nuclear fusion, meiosis and ascospore formation.

Cystidia: Distinctly shaped sterile cells present in the hymenium interspersed with the basidia in Basidiomycetes.

Dictyospore: Spores, which are multicellular in which both transverse and vertical septations are present.

Didymospore: Spores which are two celled (i.e. one septate).

Gametangium: Unicellular structure producing gametes or gametic nuclei.

Gamete: Motile or non-motile cell capable of fusion with another compatible cell (gamete) to form a zygote.

Gills: Layers of vertical tissues bearing the hymenium in agarics (also called lamellae)

Gleba: The sporulating mass inside closed sporocaps of the Gasteromycetes (Basidiomycetes).

Hymenium: Fertile spore-bearing layer in the fruit bodies of Ascomycetes (bearing asci) and Basidiomycetes (bearing basidia).

Inoperculate: Spore bearing structures lacking a specialised operculum or "cap" which opens to permit spore discharge (See Operculate).

Isidia: Protuberances from lichen talli, which may break off to serve as vegetative propagules.

Mitospore: Spore having one or more nuclei resulting from mitotic division, characteristic of asexual reproduction.

Multilocular: Fruit bodies with several internal cavities producing spores.

Oogamy: Sexual reproduction involving large non-motile female gamete (egg) and a smaller motile or non-motile male gamete, characteristic of all oomycetes.

Oogonia: Female gametangia giving rise to one or more eggs (Female gametes, which are non-motile).

Oospore: Thick walled resting spore developing from fertilised oogonium or egg of Oomycetes.

Operculate: Spore bearing structures with a specialised operculum or cap to permit spore discharge.

Ostiole: Opening at the top of fungal fruit bodies through which spores are either passively released or forcibly discharged.

Paraphyses (sing. Paraphysis): Sterile hyphae interspersed with asci in the hymenium of many Ascomycetes.

Peridiole: Discoid bodies characteristic of the "Bird's Nest Fungi" (Nidulariales, Basidiomycetes) in which the basidiospores are borne. Peridioles are splashed out of the cup-shaped basidioma by rain drops.

Peridium: Wall or limiting membrane of a fungal fruit body or a sporangium.

Perithecium: Flask-shaped or subglobose ascomata with well-defined ostiole and a regular hymenium bearing asci (see Apothecium, Cleistothecium).

Phialide: A conidiogenous cell often tapering to a point from which a basipetal succession of conidia (phialospores) are differentiated.

Podetia: Cylindrical branched structure in lichens such as *Cladonia* bearing apically the apothecial ascomata.

Puff balls: Gasteromycete basidioma in *Lycoperdon* etc. in which wind suction through the ostiole facilitates dispersal of basidiospores (see Puffing).

Puffing: Simultaneous release of ascospores or basidiospores as a visible cloud from the mature fruit bodies (e.g. Ascospores released from apothecia of Sclerotiniaceae).

Pycnidia: Flask-shaped fruit bodies of Coelomycetes with or without ostiole bearing conidia (pycnospores).

Scolecospores: Spores which are very long and thin.

Sexual reproduction: Fusion of gametes followed by meiosis and genetic recombination in the offsprings.

Sorus: Term to describe infection spots on plants in which spore aggregates burst through the host epidermis causing visible lesions on the infected parts (e.g. Rust sorus especially in the Uredial stage).

Spermagonium: Flask-shaped structure producing spermatia in rust fungi (Uredinales) life cycle, sometimes also called pycnidia.

Sporangiole: Small sprorangium, often without a columella, and with a small number of spores (Mucorales).

Sporangiophore: Specialised hyphal branch bearing one or more sporangia Oomycetes, Zygomycetes.

Sporangium: A specialised structure in which asexual spores (sporangiospores, Zoospores etc.) are developed.

Spore: Specialised microscopic propagule produced by fungi capable of developing into a new thallus without necessity of fusion with another cell.

Spore print: Visible deposit of spores discharged by Basidiomycetes fruit bodies suspended above a white surface. Spore print colour is helpful in identification of several mushrooms.

Sporocarp: Fruit bodies associated with arbuscular mycorrhizal fungi found in soils (e.g. *Glomus*)

Staurospore: Spores with radiating extensions giving star-like appearance.

Sterigmata: Short apical outgrowths from a basidium tapering to a point and bearing apically the basidiospores.

Stroma: Fungal tissue mass from which perithecia and other fructificaions are differentiated.

Suspensors: Empty walls of fusing gametangia of Zygomycetes which remain attached to mature Zygospores.

Synnema/Synnemata: Sporulating structure in some Hyphomycetes in which the conidiophores are aggregated to form rope like columns (e.g. *Isaria*).

Telcomorph: Sexual reproductive phase, especially of mitosporia fungi (see Anamorph).

Zoosporangium: Sporangia in which motile flagellated spores are differentiated and released.

Zoospore: Flagellated motile asexual spore characteristic of some aquatic fungi (Oomycetes, Chytridiomycetes)

Zygospore: Product of fusion between two gametangia in Zygomycetes.

Zygote: Diploid cell resulting from the fusion of haploid gametes.

Metabolites

Aflatoxin: A highly carcinogenic mycotoxin produced by *Aspergillus flavus* and *A. parasiticus* colonising food materials like groundnut, dry coconut, etc.

Amanitin/Amatoxins: Cyclic octapeptide toxins produced by the mushroom *Amanita phalloides,* highly toxic to humans and cause of fatal mushroom poisoning.

Amphotericin B: Polyene antifungal antibiotic derived from *Streptomyces*, used in the treatment of systemic mycoses.

Cycloheximide (Actidione): An antibacterial/antifungal antibiotic derived from *Streptomyces griseus* used in culture media for selective isolation of human pathogenic fungi.

Cyclosporine: Polypeptide derived from *Tolypocladium inflatum* selectively inhibitory to human immune system especially affecting T-cells, applied as immunosuppressant in patients undergoing organ transplants.

Ergotamine: Cylcopeptide derivative of Lysergic acid from ergot (*Claviceps purpurea*) sclerotia, used in treatment of migraine headaches.

Fermentation: Chemical changes produced by enzymes of living micro-organisms including fungi on organic substrates.

Glycogen: Insoluble carbohydrate reserve material often stored by fungi and yeasts.

Griseofulvin: Antifungal antibiotic produced by select *Penicillium* species effective in the treatment of dermatophytic infections in man and animals by oral therapy.

Immunosuppressor: Substance which can completely suppress the immune system in humans to prevent rejection of organ transplants (See Cyclosporine).

Ketjap: Indonesian fermented food-based on *Aspergillus oryzae* fermentation of black soybean.

Koji: Starter culture of *Aspergillus oryzae* and allied species grown on solid substrates like wheat or rice in the manufacture of various oriental fermented foods.

LSD: Lysergic acid diethylamide. Hallucinogenic metabolite from ergot cultures like *Claviceps purpurea*, *C. paspali*.

Meixner test: Blue colour produced by extracts from mushrooms with concentrated hydrochloric acid, indicative of amatoxins (cause of mushroom poisoning).

Miso: An oriental food product based on fermentation of cereals and soybeans with *Aspergillus oryzae* and *Saccharomyces rouxii*.

Mycotoxin: Fungal secondary metabolite having poisonous or toxic effect on man and animals.

Nystatin(Mycostatin): An antifungal antibiotic produced by *Streptomyces noursei* used in the treatment of candidiasis.

Ochratoxin: A nephrotoxic toxin produced by *Aspergillus ochracerus*.

Oestrogenic syndrome: Sexual dysfunctioning in pigs caused by the steroidal mycotoxin, zearalenone.

Patulin: Mycotoxin produced by *Aspergillus clavatus*, *Penicillium patulum* etc toxic to plants and causing neurotoxicosis in cattle.

Penicillin: Antibacterial antibiotic from *Penicillium chrysogenum* widely used in human therapy to control bacterial infections.

Penitrem: Mycotoxin affecting nervous system causing symptoms of tremor from species of *Penicillium* such as P. *cyclopium*.

Psilocybin/Psilocin: Hallucinogenic indole compound from the Basidiomycete, *Psilocybe mexicana.*

Psychedelic: Hallucinogenic compounds affecting the mind.

Shoyu: An oriental sauce of soybean and wheat fermented by yeasts, bacteria and *Aspergillus.*

Sirenin: Hormone secreted by female gamete of *Allomyces* (Oomycetes) exerting chemotactic attraction for the male gametes in high dilutions.

Slaframine: Mycotoxin produced by *Rhizoctonia leguminicola* causing excessive salivation ("Slobbering") in ruminants fed on the infected leguminous crops.

Stachybotryotoxin: Mycotoxin produced by *Stachybotrys* (Hyphomycetes) causing toxic symptoms in horses.

"Taka diastase": Fungal amylase produced by *Aspergillus oryzae* in solid-state fermentations and used as a digestive aid. First commercial enzyme technology to be patented by **Jokichi Takamine**.

Tempeh: Oriental fermented-food based on fermentation of soybeans with *Rhizopus oligosporus.*

Tremorgen: A mycotoxin inducing neurotoxicoses and tremors in man and animals (see Penitrem).

Trichotehcenes: Mycotoxins produced by *Fusarium tricinctum*, *F. sporotrichoides*, *F. poae*, and *Trichothecium*.

Zearalenone: Mycotoxin produced by *Gibberella zeae* (anamorph *Fusarium graminearum*) cause of oestrogenic syndrome and infertility in pigs (See Oestrogenic Syndrome).

APPENDIX 2

Mycological Culture Media

Fungi are isolated in pure culture and maintained on nutrient media favourable for their vegetative growth and sporulation. Under natural conditions fungi colonise diverse substrates competing for their nutrition with other microflora. Often the degradation products of native substrates by other microbes may provide the nutrients which the fungi can utilise and thus provide favourable conditions for growth and reproduction. Since different fungi have diversity in growth rate, extent of sporulation and nutritional requirements, it would be obvious that an optimal medium formulation which would be suitable for growth and sporulation of all fungi cannot exist. Based on extensive studies on in vitro fungal cultures, several media formulations—synthetic, semisynthetic and natural, have been standardised for routine applications in mycological studies. Physiological diversity of mould cultures such as osmotolerance or growth under high alkaline conditions would necessitate introduction of appropriate modifications to the standard formulations to suit individual requirements. It must be remembered that different fungi vary widely in their sensitivity to the changes taking place in the nutrient medium composition during the vegetative growth phase. For example, rapid lowering of the pH by the growing colony on sugar-rich media may lead to adverse situations, unfavourable for long-term viability as well as optimum sporulation. Proper buffering of the medium would largely offset the difficulty.

Synthetic Media

Synthetic media are chemically defined and compounded from pure inorganic salts along with a pure carbohydrate. These media are very useful to study the nutritional requirements and growth behaviour of cultures under defined and highly reproducible conditions. Such media have been recommended for giving the technical descriptions of isolates, especially in the case of highly divergent strains of biotechnologically important species of *Aspergillus*, *Penicillium* etc.

1. Synthetic Mucor Agar (grams/litre)

Composition: Dextrose—40, Asparagine—2, KH

very low concentrations of yeast extract and adjusted to pH 4.0 to discourage competition from bacterial populations.

3. Richards' Solution (grams/litre)

Composition: KNO

This medium is well-suited for cultivation of many fungi, which sporulate poorly on conventional sugar containing mycological media. We have used in our laboratory half strength YPSs medium for growth and conservation of the rare mucoralean fungus *Benjaminiella multispora* which shows feeble sporulation on media like potato dextrose agar (PDA) and malt extract glucose yeast extract peptone (MGYP) agar.

7. Potato Dextrose Agar (PDA)

(a) ATCC formula

Composition: Diced potatoes—300grams, glucose—20g, agar —15 grams, distilled water—1 litre.

Boil finely diced potatoes in 500 ml water until thoroughly cooked, filter through cheese cloth and make up to one litre. The agar is dissloved in the filtrate by heating and the glucose is added just prior to sterilisation.

(b) C.M.I. formula (Plant Pathologist's Pocket Book, 1968 p. 239)

Composition: Potato—200 grams, dextrose—20 g, agar—20 g, water—1 litre.

Scrub the potatoes, cut into cubes without peeling. Rinse 200 grams potatoes under running water and boil in one litre water until soft, mash and squeeze as much of pulp as possible through a fine sieve. Add agar and boil till dissolved. Add dextrose and stir till dissolved and make up to one litre. Agitate stock while tubing to ensure that each tube has a proportion of the solid matter. Sterilise at 15 pounds for 20 minutes.

Good results have been obtained with 0.2% dextrose. Old potatoes are preferable to new ones. 0.2 g calcium carbonate and 0.2 g magnesium sulphate may be added.

PDA is a useful all-purpose medium widely used in mycological research.

8. Malt Extract Glucose Yeast Extract Peptone (MGYP) Agar (grams/litre)

Composition: Malt extract—3, glucose—10, yeast extract—5, peptone—5, Agar—20, distilled water—1 litre.

Sterilisation at 15 lb pressure for 15 minutes.

A medium suitable for cultivation and conservation of a wide range of fungal cultures.

9. Malt Extract Agar Blakesles's Formula (grams/litre)

Composition: Malt extract—20, glucose—20, peptone—1, Agar—20, distilled water—1 litre (add glucose just prior to sterilisation).

10. Leonian's agar: (grams/litre)

Composition: Peptone—0.625, maltose—6.25, malt extract—6.25, KH

The above medium can be modified by supplementing with 10 grams per litre of tomato paste and 10 grams per litre of oatmeal.

Both the formulations have been reported to successfully induce sporulation in many weakly sporulating strains as well as those strains, which remain vegetative for prolonged periods of culture.

12. Potato Carrot Agar

Composition: Grated potato—20, grated carrot—20, agar—20, Tap water—1 litre.

Boil grated vegetables for one hour in tap water and strain through a fine sieve without squeezing the solid residues through it. Make volume up to 1 litre, add agar and boil over water bath until agar dissolves. Tube and sterilise at 15 lbs pressure for 20 minutes.

A weak nutrient medium favouring rapid onset of sporulation and well-suited for conservation purposes. Placing a sterile filter paper strip on the slant surface is recommended for cellulolytic fungi, which colonise the filter paper and develop the sporulating structures on it (e.g. *Chaetomium*, *Trichoderma*, *Stachobotrys*, etc).

13. Hay Infusion Agar

Composition: Partially decomposed hay or rice straw 50 g, tap water 1 litre. Autoclave hay and water together at 121°C for 30 minutes filter and make up to volume.

Add 2 g KH

rapidly form their characteristic fruiting structures facilitating rapid identification.

14. Oatmeal Agar (grams/litre)

Composition: Oatmeal—30, agar—20, water—1 litre.

Powdered oatmeal is boiled in water over water bath, stirring occasionally for one hour and then squeezed through muslin cloth and made up to volume (one litre). Agar is added, boiled and sterilised at 15 lbs for 20 minutes.

The medium is particularly suited for growth and conservation of *Pythium*, *Phytophthora* and many other Oomycetous fungi.

15. Corn (maize) Meal Agar (grams/litre)

Composition: Maize meal—20, agar—20, water—1 litre.

Boil maize meal in water in a sauce pan for one hour with constant stirring. Cool and filter through muslin cloth. Add agar and boil to dissolve. Autoclave at 15 lbs for 20 minutes.

This medium was widely used by Charles Drechsler in his extensive studies on saprophytic Entomophthorales as well as predaceous fungi isolated from plant litter. A 1:10 dilution of the medium has also been found to be effective in minimising the competition from bacteria in the study of predaceous fungi, especially the Zoopagales infecting amoebae.

16. Bean Pod Agar

Composition: French bean pods—50 g, tap water—1 litre.

Bean pods are homogenised with water in a blender and made up to one litre, 20 grams of agar added, steamed to dissolve the agar, tubed and autoclaved 15 lbs for 20 minutes.

We have found that this medium favours rapid sporulation and good long-term conservation of several Hyphomycetes and Coelomycetes. Species of *Benjaminiella* (Mucorales) showed extensive development of asexual spores and zygospores.

Bean pod slices autoclaved and transferred to the surface of plain agar medium (2.5% agar) and inoculated with different fungi showed rapid sporulation in the case of *Aspergillus*, *Penicillium*, *Myrothecium* and *Colletotrichum*. Revival of cultures by transferring colonised bean pod slices to fresh media was sucessfully achieved after 6–9 months in most of the cases.

17. Rabbit Food Agar

Composition: Commercial rabbit food pellets—25 g, agar—20 g, distilled water—1 litre.

Rabbit food pellets are boiled in water and then allowed to steep for 30 min. It is then filtered through cheesecloth, made up to volume (one litre) and agar added, steamed and autoclaved at 15 lbs for 20 minutes.

This medium supports growth and sporulation of a wide range of fungal cultures.

18. Rabbit Dung Agar

Composition: Rabbit dung pellets are autoclaved at 15 lbs for 20 minutes.

3–5 pellets are transferred to a large test-tube and 5–10 ml of 2% plain agar is dispensed. The tubes are plugged, autoclaved at 15 lbs for 20 minutes and slanted such that the pellets extend above the agar surface.

A good medium for coprophilous Mucorales, Ascomycetes and Hyphomycetes.

APPENDIX 3

Mycological Notes on Fungi Relevant to Biotechnology

Brief mycological notes on some of the fungi which have an established record of biotechnology applications or whose potential is just beginning to emerge have been included in this write-up.

ACREMONIUM Link

Class: Hyphomycetes

Order: Monilialaes

Members of this genus have also been described under the name *Cephalosporium*. Monographic studies by Walter Gams have established that members described under the name *Cephalosporium* should now be identified under *Acremonium*.

Species of *Acremonium* are characterised by hyaline somewhat slow growing mycelial colonies from which simple awl-shaped phialides arise either from the substrate mycelium or from ropy strands of aerial mycelium. From these usually one-celled small ovate-ellipsoidal or globose conidia are developed which are held together in a slimy drop of mucilagenous matrix.

For purpose of identification, the cultures are streaked on 2% malt extract agar plates and incubated at room temperature (26–28ºC).

Telemorphic state of *Acremonium* belongs to Ascomycete genera *Nectria* or *Emericellopsis*.

Species of *Acremonium* have been reported to be endophytic in temperate grass hosts such as *Festuca.*

A. chrysogenum is the source of the therapeutically useful Cephalosporin antibiotics (US Patent 3.082, 155, 4, 535, 155)

Can. J. Microbiol. 13:332–34,1967.

US patent 3,825,473 (Cephalosporin C production)

US patent 3,979,260 (Deacetylcephalosporin C production)

Enz. Microbiol. Technol. 6:402–4,1984 (Deacetyl Cephalosporin synthetase)

A. kilianse (= *cephalosporium acremonium*) is used in the production of alkaline protease. (*Biochem.* J. 125:1159–60, 1971)

An alkalophilic cellulolytic *Acremonium*, *A. alcalophilum* was described by **Okada et al**. (*Trans. Mycol. Soc. Jpn.* 36: 171,1993)

Acremonium strictum (ATCC 34063) cause of jet fuel biodeterioration.

ACTINOMUCOR Schoet

Order: Mucorales

Taxonomy (**Benjamin & Hesseltine**, *Mycologia* 49:240–49,1957)

A monotypic genus of the Mucoraceae characterised by differentiation into stolons and verticillate sporangiophores which arise mostly from the rhizoids. Zygospores are not known.

A. elegans is the species widely distributed in the temperate and subtropical soils of the world. It has been reported to occur in natural soils up to depths of 30 cms and stimulated by NPK fertilisers in cultivated soils. Species of *Actinomucor* have also been regarded as xenotolerant based on their water requirements for growth. They can utilise a wide range of

carbohydrates and also inorganic nitrogen sources (nitrates, nitrites, ammonium and urea). In submerged culture, the mass doubling time has been estimated to be 4 hours.

Significant protease and phosphatase production in solid state fermentation has been reported. On moist wheat bran (50–63% moisture content) and 32°C, pH 7.0, significant protease activity was obtained (**Kirchhoff**. *Dissertation Abstracts* 20:1547,1959).

Actinomucor elegans (*ATCC* 6476) is used in the production of steroids by conversion (*JACS* 30:3382–89,1958).

A. elegans (ATCC 22963) is used in the production of acid protease in soybean food fermentation (*Appl. Microbiol*. 27: 906–11,1974).

A. elegans (ATCC 46124) is used in the production of sufu (*Mycologia* 57:149–97,1965)

ALTERNARIA Nees

Class: Hyphomycetes

Family: Pleosporaceae

Widespread in distribution, plant pathogenic species like *Alternaria solani* on potato and *A. brassicae* on mustard and other crucifers cause serious infections on their respective hosts. Saprophytic forms like *A. tenuis* have been isolated from soil.

Mycelium, conidiophores and conidia are light or dark brown, conidiophores simple with geniculate extensions on which the conidia are formed. Conidia are ovoid or ellipsoidal with a broad rounded base and tapering apical beak, muriform with several transverse and longitudinal septa.

Alternaric acid is a metabolite of *A. solani,* which inhibits spore germination in some fungi and causes wilting and necrosis in higher plants.

A. alternata (ATCC 36376) produces the mycotoxin tenuazonic acid when grown on cottonseed yeast extract sucrose broth (*Appl. Env. Microbiol*. 34:155–57, 1977).

Production of the anticancer drug vinblastin from an endophytic *Alternaria* associated with *Vinca* plants has been reported from China (*Chem. Abstr.* 129:172900–X, 1998).

ASPERGILLUS Link

One of the most widely distributed and heavy sporing hyphomycete (Mitosporic fungus) with immense biotechnology potential.

It is characterised by the presence of erect unbranched conidiophores which terminate in a vesicle covered with a layer of phialides (sterigmata) (Uniseriate) or have a layer of subtending cells termed metulae which in turn bear whorls of phialides (Biseriate). The metulae and phialides are produced synchronously and the phialides produce basipetal chains of conidia.

K.B. Raper and **D.I. Fennell** (The genus *Aspergillus*, Williams and Wilkins Co., 1965) monographed the genus and gave a detailed account of species descriptions and diagnosis.

Species of *Aspergillus* are widely distributed in soil, compost, decaying plant detritus and stored grain, particularly in warmer climatic conditions.

Species belonging to the *A. glaucus* and *A. restrictus* group are osmophilic while *A. fumigatus* is thermotolerant and having pathogenic implications for humans and animals by way of lung infestations by spores.

Aspergillus anamorphic state is associated with different teleomorphic genera including *Emericella*, *Eurotium*, *Fennellia* and *Neosartorya*.

In *Emericella*, *Aspergillus nidulans* is the anamorphic state and the conidiophores are biseriate producing conidia often in columnar structures. The ascomata are surrounded by

thick-walled hulle cells and the ascopores inside the cleistothecia are red to purple.

Aspergillus flavipes is the anamorphic state of *Fennellia* and the ascomata develop within masses of thick-walled elongated to helical cells.

Ascomata of *Eurotium* are yellow with a smooth wall of a single layer of cells. The conidia are often borne on uniserate sterigmata directly from the vesicle terminating the conidiophore.

In *Neosartorya*, the anamorphic state is *A. fumigatus*. The ascomata appear pseudoparenchymatous with wall layer several layers thick. The conidia are borne on sterigmata directly arising from the vesicle (uniseriate conidiophores).

Species of *Aspergillus* have been found to be most valuable for the manufacture of different metabolites by fermentation. Notable examples include citric acid from *A. niger*, itaconic acid and the therapeutically useful hypocholestemic agent Lovastatin from *A. terreus*. Several industrially useful enzymes are manufactured with the aid of *Aspergillus* strains. Examples include alpha amylase, ("Taka diastase" from *A. oryzae*) amyloglucosidase, acid stable protease and alpha galactosidase from *A. niger*.

Antifungal antibiotics like aculeactin from *A. aculeatus* and Echinocandin A from *A. nidulans* var. *echinulatus* are other valuable metabolites obtained from aspergilli.

Aspergilli are also the sources of very powerful mycotoxins like aflatoxins produced from *A. flavus* and *A. parasiticus*, being a highly potent liver carcinogen contaminating food materials and animal feeds to cause serious health problems.

Sterigmatocystin, a carcinogenic hepatotoxin and a precursor of aflatoxin B

Colonies are slow growing attaining a diameter of 2 cm in 10 days, white turning pale yellow or pinkish with age. Conidiogenous cells arise singly or in whorls from the vegetative mycelia, inflated at the base and bearing an acropetal succession of spores on an elongated rachis which appears zigzag or tortuous. Conidia are globose, subglobose or ellipsoidal, hyaline with a rounded or somewhat protuberant base (Description based on *B. bassiana*).

Beauveria is the anamorphic state of the teleomorph *Cordyceps* (Ascomycete).

B. bassiana is the most widely distributed species on insect populations belonging to lepidoptera, coleoptera and diptera.

Mass sporulation and application of the spores for biocontrol of insects has been investigated and *B. bassiana* sporulates best on sucrose or glucose media containing asparagine and supplemented with yeast extract.

Proteolytic and lipolytic activities have been demonstrated and the lipolytic activity correlates with the virulence of the strain during pathogenesis.

Some of the metabolites from *Beauveria* investigated include a red pigment Oosporein, and yellow pigment Tenellin and a cyclodepsipeptide, beauverilide.

CHAETOMIUM Kunze

Class: Ascomycetes

Family: Chaetomiaceae

Widely distributed in soil, plant litter and dung and can be readily isolated from these substrates. The ascomata are subglobose to vasiform covered prominently with branched, septate and highly contorted hairs, which give a very characteristic appearance to the fruit bodies. The asci which are borne inside the ascomata are thin-walled, deliquescent and releasing ellipsoidal to lenticular shaped thin-walled ascospores. Most species are homothallic and lack a conidial

state, while in some heterothallic species an *Acremonium* like anamorphic state has been reported.

Chaetomium is an important genus known for its ability to degrade cellulosic and lignocellulosic substrates and plays an important role in the recycling of lignocellulosic plant residues back to the soil.

Chaetomium cellulolyticum has been identified as a species capable of producing microbial biomass product for the protein supplementation of animal feeds by virtue of its ability to metabolise and grow well on alkali cooked cereal straw substrates.

Good growth and rapid sporulation are obtained on cellulose or starch containing media supplemented with malt extract, yeast extract or potato carrot extracts.

Potato carrot agar media with filter paper strips added is very suitable for conservation of *Chaetomium* cultures in vitro.

CONIDIOBOLUS Brefeld

Class: Zygomycetes

Order: Entomophthorales

Species of *Conidiobolus* are widely distributed in association with decaying plant detritus and are isolated by overlaying finely sieved detritus particles moistened with soft agar or water above agar media.

The conidia which are forcibly discharged on to the agar medium readily develop bacteria-free colonies, which can be isolated in pure culture. The vegetative mycelium is broad, aseptate when young but with withdrawal and localisation of cellular contents become differentiated into hyphal segments. The asexual reproductive phase which develops in young cultures consists of phototrophic unbranched conidiophores which bear globose conidia at the apex. These at maturity are discharged with force, often falling beyond the growing edge of the colony, readily germinating to give rise to daughter

colonies. Conidia germinate by germ tubes or give rise to secondary globose conidia by evagination of the contents or in some species like *C. coronatus* give rise to small microconidia on radial sterigmata. Formation of elongate conidia which are forcibly discharged are known in three species, while in some others like *C. heterosporus* elongate conidia are borne on filamentous conidiophore and passively liberated. Zygospore formation in the sexual phase consists of fusion between anisogamous gametangia.

Conidiobolus species are homothallic and in the zygospore forming species, mostly smooth walled. Species like *C. utriculosus* or *C. rhysosporus* have warty or undulate zygospore walls. The zygospores are characterised by a large globose reserve body in the centre.

Some species of *Conidiobolus* have entomopathogenic tendencies and some have been isolated as opportunistic infections in man and animals.

Good growth and sporulation occurs in media containing sugars like glucose or maltose supplemented with organic nitrogen sources.

Most of the species are highly proteolytic and strains of *C. coronatus* isolated from plant detritus have been identified capable of secreting high levels of alkaline protease with potential applications in leather and detergent industries (*Biotechnol. Lett*. 5:285–88, 1983, *Enz. Microbial Technol*. 15: 72–76, 1993). The protease has also been successfully tried out in animal cell cultures as an alternative to conventional trypsin (*Biotechnol. Lett*. 7: 665–68, 1985).

Conidiobolus cultures have also been studied for production of polyunsaturated fatty acids in Japan and several patents have been granted for the production of PUFA such as gamma linolenic acid and docosahexaenoic acid.

CUNNINGHAMELLA Matr

Class: Zygomycetes

Order: Mucorales

Colonies fast growing on mycological media, at first white but later turning gray, turf in sporulating cultures up to 3 cm high. Sporangiophores are often verticillately branched terminating in globose or subglobose vesicles bearing sporangioles with well-defined spines. Many species are heterothallic and zygospores from mated cultures are globose, brownish with pointed tuberculate projections.

C. blakesleeana is the best known species.

Species of *Cunninghamella* are widely distributed in tropical and subtropical soils. *C. echinulata* is a species frequently recovered from Indian soils.

Strains of *Cunninghamella* have been studied for stereospecific biotransformation of some steroids but have not been explored intensively for their biotechnology potential.

CURVULARIA Boedijn

Class: Hyphomycetes

Family: Dematiaceae (Teleomorph: *Cochliobolus*)

Widespread in soils and associated with decomposing plant materials, some strains are weak plant pathogens, often encountered in seed testing being seed borne and readily develop in seed samples incubated on moist blotters. Mycelium dematiaceous, spreading, growing reasonably rapidly on mycological media. Conidiophores ascendent, pigmented, geniculate developing large single conidia through conspicuous pores. Conidia multiseptate, curved or fusiform, 3 or more septate and with the end cells paler than the rest of the central cells in the spore.

C. lunata is the most common species and presently about 35 species are recognised, mostly subtropical or tropical in distribution. *C. lunata* has been used for steroid transformation studies, for example, hydroxylation of cortexolone to hydrocortisone (*Appl. Env. Microbiol.* 45:436–43,1983).

EMERICELLOPSIS J.F.H.Beyma

Class: Ascomycetes

Family: Trichocomaceae

Order: Eurotiales

Species of *Emericellopsis* are homothallic and form the ascomata readily in culture. The nonostiolate cleistothecia have wall layers several cells thick composed of hyaline flattened cells. Asci are globose and contain eight ascospores, which are very characteristic with longitudinal gelatinous ridges.

E. minima and *E. terricola* are the most common species known.

The associated anamorph state generally belongs to the genus *Acremonium* (= Cephalosporium).

Emericellopsis synnematicola described from India has a *Stilbella* anamorphic state (*Mycologia* 52:695,1960). This species produces the antiprotozoal, antihelminthic antibiotic antiamoebin (US Patent 3,657,419).

E. minima produces emerimicin complex of antiprotozoan antibiotic (US Patent 3,821,367) (*J. Antibiotics* 27:274–82,1974).

E. minima (*E. salmosynnemata*) also produces the antibiotic Cephalosporin N (Synnematin B).

Temperature optima is around 30°C and growth can occur up to 37°C.

pH optima vary between 5.0 and 7.0 and the cultures are capable of utilising various carbon sources including glucose, sucrose and maltose with inorganic nitrogen sources and supplemented with biotin. Oat meal agar and corn meal agar supported abundant cleistothecial development, particularly in fresh isolates.

FUSARIUM Link

Class: Hyphomycetes.

Anamorphic state of Hypocreaceae (Ascomycetes)

Teleomorphs : *Nectria, Gibberella, Calonectria,* etc

An important genus comprising saprobes and plant pathogens. Characterised by usually fast growing pale or bright coloured colonies, with an aerial mycelial felt and sporulation often in well-differentiated sporodochia.

The conidiophores are branched at the base and occurring in well-defined pustules (sporodochia) or diffuse confluenting pionnotes or borne at the tip of phialides on erect branches and aggregated together into slimy droplets.

The macroconidia are fusiod, often curved and multiseptate while rounded unicellular microconidia are produced in slimy droplets or borne in moniliform chains (*F. moniliforme*).

Formation of chlamydopores often in chains in the vegetative mycelium is a common feature in many *Fusarium* species.

Taxonomy and precise species identification of fusaria is a highly specialised area and several schemes of classification have been put forward by various authorities like **Wollenweber** and **Reinking**, **Snyder** and **Hanson**, **Booth**, etc.

Species of *Fusarium* are widely distributed in soils and these include the saproboic forms as well as the plant

pathogenic species which have a high degree of competitive saprophytic ability and survival in the saprophytic phase.

Fusarium oxysporum var. *vasinfectum* causes serious wilt disease of cotton, var. *udum* causes chickpea wilt and banana wilt is caused by var. *cubense.*

Fusarium moniliforme (Telelomorph: *Gibberella fujikuroi*) is the species employed for the manufacture of gibberellic acid and the gibberellins.

Many mycotoxins are produced by *Fusarium* species, notable among which may be mentioned zearalenone from *F. graminearum* (telelomorph: *Gibberella zeae*), trichothecenes from *F. semitectum* etc.

GEOTRICHUM Link

Anamorph of Dipodascaceae (ascomycetes)

Telelomorph : *Dipodascus*, *Galactomyces* etc.

Geotrichum candidum (also referred to as *Oidium lactis*) is isolated from milk.

The genus consists of hemiascomycetes with arthroconidia formed by segmentation and release of the individual cells and naked globose to elongate asci arising from conjugation of gametangial cells.

The anamorphic *G. candidum* is characterised by creeping, mostly submerged septate hyphae, which readily undergo fragmentation into arthroconidia which remain cylindrical or become barrel shaped or ellipsoidal. The colonies are fast growing bearing the arthroconidia in large numbers on erect or decumbent aerial branches. *G. candidum* is worldwide in distribution and can be isolated from soil, water, sewage etc. It also forms a sour rot of citrus fruits and very frequently associated with milk and milk products. A selective isolation procedure based on its tolerance to novobiocin and carbon dioxide has been published (*Mycologia* 54:106–109,1963). Conditions for arthroconidia formation depend on carbon,

nitrogen balance in the culture media and in heavy density spore populations, self-inhibition of spore germination is often observed.

A wide variety of carbohydrates are utilised as growth substrates and addition of vitamins is necessary for growth. Strong lipase production was observed in media containing rice bran, olive oil, oleic or linoleic acids (*Agric. Biol. Chem*. 37: 929–31,1973)

GLIOCLADIUM Corda

Mitosporic fungi, Teleomorph: *Nectria*, *Hypocrea* (Ascomycetes)

The hyphomycetous genus *Gliocladium* is characterised by densely penicillate conidiophores bearing slimy one-celled conidial heads or conidia borne in columns. The limits of the genus are not clearly defined and the genus is regarded as having affinities with *Penicillum*, *Verticillium*, etc. The diagnoistic features of the genus may be defined as fungi with penicillate heads producing spores in slime balls or loose columns.

The genus *Clonostachys* characterised by ellipsoid conidia in columnar chains was regarded as distinct from *Gliocladium*, but recent concepts tend to consider it as synonymous with *Gliocladium*.

G. roseum occurs in soil as well as rotting plant debris and due to its abundant formation of phialoconidia, the cultures are easily isolated by dilution plate and other techniques. It has often been found in birds' nests, wood pulp or filter paper exposed in a river and organic debris in soil.

G. roseum (ATCC 8684) and *G. virens* (ATCC 13362) produce L-DOPA from L-Tyrosine and its derivatives (US patent 3,671,397).

Some strains of *G. virens* have been studied as biocontrol agents against *Sclerotinia minor* and *Sclerotium rolfsii* (*Phytopathology* 74:1171–75,1984).

HUMICOLA Traaen

Mitosporic fungi.

The vegetative mycelium is hyaline to slightly pigmented and is differentiated into conidia on short lateral branches functioning as undistinguished conidiophores. The conidia bearing structures may show slight inflation at the apex and develop a subglobose or globose, smooth-walled unicellular aleuriospores which are dark brown in colour.

A closely related genus *Thermomyces* which is thermophilic and bear single conidia with roughened walls and warty outgrowths, has been regarded by some taxonomists as identical with *Humicola*. Presently the industrially important *Humicola lanuginosa* is grouped under *Thermomyces* and called *Thermomyces lanuginosus*.

Species of *Humicola* are widely distributed in soils with neutral or alkaline pH and are more readily isolated on media containing cellulosic substrates, since many strains are strongly cellulolytic.

Humicola/*Thermomyces* strains have been extensively studied for the production of lipase, particularly the alkali-stable commercial enzyme designated Lipolase which is compatible with detergents and effectively removes fat induced stains from fabrics. In a series of patents filed by Novo Nordisk on this lipase, variants developed through sophisticated recombinant techniques have been described (*Chem. Abst.* 121: 136684, 1994, 125:52380,1996). Heterologous production of the lipase variants with better compatibility and wash performance have been successfully achieved in *Aspergillus oryzae* (*Chem. Abst.* 122:155213,1995, 131:256405,1999).

Humicola insolens strains have been identified producing cellulase, which are optimally active at 60°C and pH 5–10 (*Chemical Abstracts* 121:249074,1994). Xylanases from *Humicola* have also been cloned and partially sequenced and the enzyme produced in *A. oryzae*. This enzyme was found to be useful in baking industry as well as pulp and paper bleaching. Extracellular thermostable alpha galactosidase production from *Humicola* has also been reported (*Chemical Abstracts* 123:222445,1995).

MORTIERELLA Coemans

Class: Zygomycetes

Order: Mucorales

Family: Mortierellaceae.

The genus *Mortierella* is characterised by delicate mycelial colonies bearing sporangia which lack columella and may spilt into numerous, few or single spores.

Two subgroups of the genus have been recognised:

(a) *Micromucor*, characterised by rather slow growing velvety colonies, develop somewhat pigmented sporangia on 2% malt extract agar.

(b) *Mortierella*, having thin spreading mycelium with hyaline sporangia and garlic-like odour. For this group, sporulation is optimal either on soil extract agar or potato carrot agar. On malt extract agar, the abundant aerial mycelial growth obscures the sporangiophore development.

Many species form chlamydospores, sometimes with ornamentations and these have also been designated as stylospores.

M. epigama and *M. chlamydospora* are homothallic and others like *M. parvispora* are heterothallic and the zygospores in either case are associated with unequal suspensors.

Zygospore formation is favoured by media like potato carrot agar or hempseed agar and incubation is preferably at 18–20°C.

Mortierella alpina strains have been widely studied for the production of gamma linolenic and arachidonic acid by fermentation. *M. vinacea* (ATCC 20034) produces an alpha galactosidase and the enzyme is used for removing raffinose and stachyose from soybean milk (US patent 3,647,625, *J. Food Sci*. 41:173–75, 1976).

MUCOR Fresen.

Class: Zygomycetes

Order: Mucorales

Family: Mucoraceae

The family Mucoraceae is characterised by multispored sporangia borne on sporangiophores and the sporangia have distinct columella.

Colonies of *Mucor* are rapid growing producing a conspicuous aerial turf of mycelium. The sporangia mature rapidly imparting a grayish colour to the colony at maturity.

Sporangiophores are without basal rhizoids (as in *Rhizopus*) sometimes sparingly branched and bearing many spored sporangia having a well-developed columella. Spores are released at maturity by rupture of the sporangial wall and appear hyaline or light coloured, smooth or with fine ornamentations, globose to ellipsoidal in shape.

Some *Mucor* colonies give rise to yeast phase development in the submerged hyphae. When inoculated into sugar-rich media in broth and incubated under stationary conditions, differentiation of yeast cells (rounded unicellular bodies) rapidly settling at the bottom are observed. Such species are termed dimorphic (e.g. *M. rouxii*).

Mucor species are generally heterothallic and zygospores are produced in the aerial mycelium between compatible mating types when inoculated on suitable media. They appear dark brown, often with stellate warts.

Mucor cultures have been studied for their production of extracellular lipase, protease and also for the production of polyunsaturated fatty acids. Gamma linolenic acid production in *Mucor circinelloides* was enhanced in the presence of acetate in a medium containing sunflower oil (*Syst. Appl. Microbiol.* 22:155–60,1999). Lipase production was studied in soyflour, sucrose, olive oil medium and maximum activity was obtained at 96 hours and the lipase was stable to pH7–10 and showed maximum activity at 50°C (*Chem. Abst.* 130:167196,1999).

MYROTHECIUM Tode ex Fr.

Mitosporic fungi.

Species of *Myrothecium* especially *M. verrucaria* are well known as cellulolytic saprophytes, while *M. roridum* is a weak plant pathogen causing leaf lesions on various host plants. The spores are borne on well-defined sprodochia which appear as dark cushion like or cupulate structures bearing numerous spores in blackish to olive green slimy masses. Individual spores are hyaline to olive brown and ovoid, fusoid or cylindrical in shape.

Sporulation on media like corn meal agar or oat meal agar takes place after 10–15 days and is stimulated by light. Lupin stems or autoclaved grass leaf are readily colonised and the sporodocia readily develop on these natural substrates. Optimal temperature for growth on agar is between 25–27°C, while a broad pH range is tolerated by most isolates. Growth and sporulation were optimal with D-glucose and with various nitrogen sources, notably nitrates, urea and glutamic acid.

Several toxins like myrothecin, roridin and necrocitin have been isolated which induce wilt symptoms in tomato seedlings.

Necrocitin isolated in India (*Hindustan Antibiotics Bull*. 8:59–63,1965) has also been shown to be toxic to snails. Macrocyclic trichothecenes isolated from *M. Verrucaria* have been shown to have antimalarial activity (*Chem. Abst*. 130:148268,1999).

Extracellular chitinase from *M. verrucaria* was shown to be an antagonist to the groundnut rust, *Puccinia arachidis* (*Chem. Abst*. 130:77791,1999).

Cellulose degradation by *Myrothecium* is extensive but the level of extracellular enzyme secretion is low compared to fungi like *Trichoderma* or *Penicillium* strains.

PENICILLIUM Link ex Fr.

Class: Hyphomycetes

Order: Eurotiales

Telelomorph: *Talaromyces*, *Trichocoma.*

The genus is characterised by conspicuous conidiophores which are erect, branched or divergent near the apex or adpressed to the conidiophore axis, generally brush-like in appearance.

The basipetal conidia are borne on dry chains arising from flask shaped phialides (which are sometimes referred to as sterigmata). The degree and extent of branching of the spore-bearing structures has been key factor in the taxonomy of penicillia, classifying them under monoverticilliate, biverticilliate, etc.

Colony characters are studied on either Czapek-Dox agar or 2% malt extract agar and the vegetative growth of the colony and the sporulation behaviour are helpful in differentiating the various subgroups under which penicillia are classified.

Species of *Penicillium* are ubiquitous and widely distributed in soils and also in association with decomposing plant litter.

Some like *P. digitatum* cause storage rots in citrus fruits, especially orange.

The genus *Geosmithia* was separated from *Penicillium* by **Pitt** (*Can. J. Bot.* 57:2021–30,1979) based on the following characters: (a) colonies with conidia in colours other than grey-blue or grey-green; (b) penicilli with all elements roughened and with both the phialides and conidia cylindrical. The conidia are formed from terminal pores and not from tapering collula (necks) characters of penicillia. *Talaromyces* is the teleomorphic state of this genus also and *T. emersonii*, a thermophilic cellulolytic fungus has its anamorphic state as *Geosmithia emersonii.*

Biotechnologically, species of *Penicillium* are very important. Apart from well-established antibiotics like penicillin and griseofulvin, useful metabolites like compactin with anticholestimitic activity produced by *P. brevicompactum* are important.

P. funiculosum and *Talaromyces emersonii* are capable of secreting high levels of extracellular cellulase activity to hydrolyse solid cellulosic substrates. *P. camemberti* and *P. roquefortii* find widespread application in the manufacture of cheeses with specific flavours.

Mycotoxins produced by penicillia include islandicin by *P. islandicum* a cause of hepatitis (yellow rice disease) in humans and verruculotoxin produced by *P. verruculosum*.

RHIZOMUCOR Lucet & Costantin

Class: Zygomycetes

Order: Mucorales

The genus is distinguished from *Mucor* by the presence of rhizoids at the base of the sporangiophores and the thermophilic behaviour of its species. The wide sporangiophores are branched sympodially and often show umbellate branching in the apical region. The sporangia are

columellate, spinulose and rupture at maturity. Zygospore formation is similar to *Mucor*. *R. miehei* and *R. pusillus* are the two best-known species (which were formerly called *Mucor miehei* and *M. pusillus* respectively). While *R. meihei* is regularly homothallic, *R. pusillus* is generally heterothallic.

R. pusillus is widely distributed and is mainly isolated from composting and fermenting substrates such as garden compost, municipal wastes, cultivated mushroom beds, etc. It has also been isolated from leaf mould, birds' nests, grassland soils and peat.

Both the species are themophilic and grow optimally at around 50°C. A wide range of carbohydrates are utilised by either species for growth. Inorganic nitrogen sources as well as several amino acids can meet the nitrogen requirements. Moderate attack on cellulose and formation of endo 1,4-beta glucanase has been reported.

Commercial scale manufacture of an acid protease useful in the manufacture of cheese (milk clotting enzyme) has been carried out with strains belonging to both the species.

RHIZOPUS Ehrenb.

Class: Zygomycetes

Order: Mucorales

Colonies are very fast growing forming hyaline, aerial stolons and sporangiophores bearing columellate sporangia. Pigmented rhizoids occur in defined nodes, which is characteristic of the genus.

The sporangia bear numerous spores which are ellipsoidal often with pointed ends, sometimes showing striations. Species of *Rhizopus* are either homothallic or heterothallic.

Many of the species of *Rhizopus* are technologically important. For example, *R. azygosporus* and *R. oligosporus* strains are used in the manufacture of the Indonesian

fermented food "tempeh". Pectolytic enzymes are produced by *R. stolonifer* and *R. circinans* while strains of *R. oryzae* (*R. arrhizus*) are sources for the manufacture of fumaric acid. Production of laminarinase and ß-1, 3 glucanase by *R. oryzae* (ATCC 24563) has been reported.

Rhizopus species are widely distributed, especially in the tropical and subtropical regions. Many species are associated with soft rots of fruits and tuberous crops such as potato, sweet potato, etc., by virtue of their ability to degrade pectin. Some strains are also employed for the commercial manufacture of amyloglucosidase, which is involved in the enzymatic conversion of starch to glucose in association with bacterial alpha amylase.

TRICHODERMA Pers.

Class: Hyphomycetes

Teleomorph : *Hypocrea* (Ascomycetes)

The genus is characterised by fast growing hyaline colonies in which the conidiophores are repeatedly branched bearing flask-shaped phialides on which conidia are borne in large numbers agglutinated into tiny slime drops.

Actively sporulating colonies have a greenish or greenish-yellow colour and it is established that light stimulates sporulation. In cultures periodically exposed to light alternating with incubation in the dark, concentric zones of heavy sporulation corresponding to the period under illumination can be observed.

The conidia are single-celled, hyaline but appearing greenish in mass, smooth-walled or roughened in some species.

T. saturnisporum has characteristic wing-like extensions to the spore walls.

Trichoderma cultures are grown on oat meal agar, malt extract agar or media containing cellulosic substrates such as

solka floc. They are best grown in diffuse light or under Near ultraviolet light since many strains grown continuously in the dark tend to lose the ability to sporulate.

Trichoderma species, especially *T. reesei* have been extensively investigated for their ability to produce extracellular cellulolytic enzymes, particularly having high levels of cellobiohydrolase, which is the key enzyme for the ability to break down solid cellulosic substrates and hydrolyse it to cellobiose and glucose. *Trichoderma* cultures are generally poor in extracellular β-glucosidase secretion.

Mutants of *Trichoderma* secreting high amounts of soluble protein have been identified, some of which have been successfully employed for heterologous protein secretion based on cloned genes.

T. viride has been extensively investigated as a biological antagonist and practical technologies for the field control of root disease fungi on crop plants such as *Rhizoctonia solani* and *Pythium* sp. have been successfully achieved.

APPENDIX 5

Some Mycological Techniques

Handling of Field Collections

An important aspect of natural fungal diversity exploration is the collection of the specimens of fungi from the field and establishing in vitro pure cultures. Mushrooms and other microfungi can be readily collected during the monsoon months. Careful examination with a hand lens of natural substrates such as dung, plant litter, wood, bark, etc., would reveal sporulating fungal colonies. During transportation to the laboratory, utmost care is necessary to maintain the specimens as close to their natural humid environment as possible.

Fleshy fungi can be transported in banana sheaths, which are folded from two sides to form a packet. The specimens remain fresh and viable without drying or degeneration. For small microfungi on natural substrate, transfer them individually in small polythene bags or specimen tubes and these are transported to the laboratory by placing them inside self-sealing ("Zip-loc") plastic bags in which moistened cotton swabs are placed. The high humidity provided by the condensed moisture droplets ensures the necessary environment for the specimens to remain fresh and viable. Insect pathogenic fungi carefully transferred into the specimen tubes and placed in the plastic bags as indicated

above also gave excellent results in establishing successful cultures.

Microscopic Study of Fungal Sporulation

For proper identification of many fungi, the morphology of the conidiophores and the manner in which conidia are differentiated are required to be studied in detail. Slides prepared from fully sporulated cultures often fail to reveal the developmental stages of conidiophore and conidial morphogenesis.

The following simple technique enables the follow-up of sporulation patterns, especially in the Hyphomycetes fungal cultures:

An agar medium suited to support active sporulation is poured in petri plates. After the agar solidifies, 8–10 pieces of cellophane squares (plain transparent non-wettable cellophane) autoclaved in water are transferred on the agar in each plate. A small blob of soft agar (1%) mixed with the spores is transferred to the centre of each cellophane square and the plates are incubated at temperatures optimal for growth and sporulation.

The spores germinate forming a mycelial colony and subsequently sporulation is initiated. Periodic observation to follow up the sporulation process and preparation of slides is easily accomplished. At different intervals, a cellophane square with different stages in sporulation is mounted in lactophenol cotton blue and the coverslip edges are sealed with nail varnish. By sequential study of the various slides prepared, a progressive picture of conidiophore morphogenesis and conidial development can be studied. Inclusion of a thin section of an autoclaved grass leaf on the cellophane square has been found to be beneficial. The sporulating structures colonise and anchor to the grass leaf section and remain intact during the process of slide preparation.

Lactophenol-Cotton blue Mounting Medium has the following composition:

Phenol—20 g, Lactic acid—20 g, Glycerol—40 g, Water—20 ml.

A dilute solution of a stain like cotton blue is added and stored in stoppered bottles.

Bacterial Contamination in Stock Cultures of Fungi: Detection and Purification

Stock cultures of fungi, if contaminated by bacteria cannot be easily detected by microscopic examination or staining. This is particularly true in the case of fungal colonies with a well-developed turf of aerial mycelia and conidiophores as in the case of mucoralean fungi. The best way to ascertain purity of the stock cultures is to transfer a portion of the culture including the basal agar medium to tubes of nutrient broth media such as MGYP broth and incubating on the shaker for 48 hours. If there is a bacterial contamination, the broth will turn cloudy due to bacterial growth, otherwise there will be only mycelial growth and the broth will remain clear.

In order to purify, a spore suspension in sterile water is streaked on a nutrient agar medium supplemented with 25 μg/ml of a broad-spectrum antibacterial antibiotics like tetracycline. Individual bacteria-free colonies are subcultured and after growth, tested again by inoculating portions of the colony into fresh MGYP broth to confirm the absence of bacteria in the subcultures.

Prevention of Mite Infestation in Stock Cultures

Mites are very tiny insects, which invade culture tubes, graze on the cultures and contaminate them and as such pose very serious threats in the conservation of pure cultures for biotechnological work. The source of the mites can be from natural materials brought into the laboratory for study and one of the most important strategies should be to maintain high level of cleanliness in the culture area. It is advisable to spatially separate the area in which field materials are handled from the culture zone earmarked for maintenance and subculture of stocks. One of the suggested methods for minimizing risk of mite infestation has been as follows:

The cotton wool plugs of culture tubes are pushed well down and the tube-rim heated gently and coated with copper sulphate gelatin adhesive (copper sulphate—20%, gelatin—20% and water—60%). Over this a covering of cigarette paper is placed and after the paper has stuck, excess cigarette paper is burnt off. The paper is porous to permit passage of air while preventing mite penetration.

APPENDIX 6

International Depository Authorities for Fungal Cultures

The meaningful translation of naturally occurring microbial diversity for biotechnology innovations are facilitated by their availability in pure cultures conserved in germplasm banks ensuring genetic stability. Global recognition of its importance is evident from the formation of the World Federation of Culture Collection (WFCC) and the World Data Centre of Micro-organisms (WDCM). The WFCC is a multidisciplinary commission of the International Union of Biological Sciences (IUBS) and Federation within International Union of Microbiological Societies (IUMS). The WFCC is concerned with the collection, authentication, maintenance and distribution of cultures of micro-organisms and cultured cells. According to the home page of culture collections in the world (updated May 1, 2003) there are 462 culture collections in 62 countries registered with WDCM and this may be consulted for details on various culture collections (see http:// wdcm.nig.ac.jp/ Details of some of the important culture collections maintaining fungal cultures and/or recognised as International Patent Depositories are given below:

1. American Type Culture Collection (ATCC)
Home Page: http:// www.atcc.org
Postal Address: 10801, University Boulevard, Monassas, VA20110-2209, USA.
Correspondent: Cypess, Prof. Dr. Raymond H.
email: rcypess@atcc.org

2. CABI Bioscience Genetic Resource Collection, CABI Bioscience UK centre (Egham) (IMI) Home page:
http:/ www.cabi.org
Postal Address: Bakeham Lane, Egham, Surrey TW209TY, UK
Correspondent: Dr. David Smith
email: dsmith@cabi.org

3. Centraalbureau voor Schimmelcultures Fungal and Yeast Collection (CBS)
Home page: http:// www.cbs.knaw.nl
Postal Addres: Uppsalalaan, 83584 CT Utrecht,
P.O. Box 85167, 3508, UTRECHT, Netherlands
Correspondent: Dr. J.A. Stalpers
email: info@cbs.knaw.nl

4. Japan Collection Of Microorganisms, RIKEN (The Institute of Physical and Chemical Research (JCM)
Home page: http:// www.jcm.riken.go.jp/
Postal Address: Hirosawa , Wako , Saitama 351 – 0198, Japan
Correspondent: Dr. Masako Takashima
email: curator@jcm.riken.go.jp

5. Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology (MTCC)
Home Page: http://www.imtech.ernet.in/mtcc
Postal Address: IMTECH, Sector 39A, Chandigarh,
U.T.160 036, India
Correspondent: Dr. Tapan Chakrabarti
Fax: (91) 172-690632, (91) 172-6905

6. National Collection of Industrial Microorganisms, (NCIM)
Postal Address: Biochemical Sciences Division,
National Chemical Laboratory, Dr. Homi Bhabha Road,
Pune 411 008, India
Correspondent: Dr. D.V. Gokhale
email: ncim@ems.ncl.res.in

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Index of Technical Names

Subject Index

Fig. 1 ***Acremonium***
Conidia borne in slime drops on short conidiophores arising from aerial mycelium.

+CMYK

Plate 2

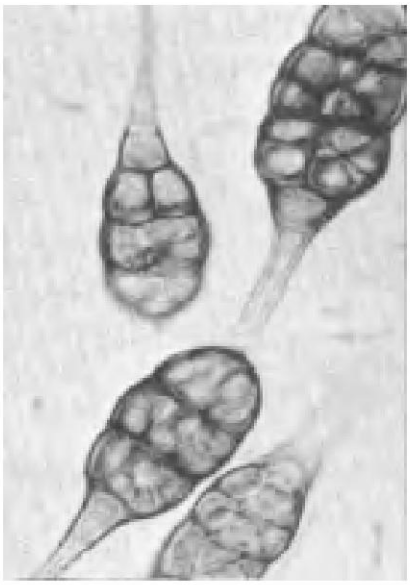

Fig. 2 ***Alternaria***
Muriform multiseptate conidia

Fig. 3 ***Aspergillus giganteus***
Claviform conidial head with club-shaped vesicle bearing uniseriate sterigmata and conidia.

Fig. 4 ***Aspergillus fumigatus***
Conidiophores showing apical vesicles bearing conidial chains.

Fig. 5 ***Aspergillus niger***
Colonies on MGYP agar showing blackish masses of conidiophores and conidial development.

+CMYK

Fig. 6 ***Conidiobolus coronatus*** Colony growth on MGYP agar showing powdery masses of conidia covering the colony surface.

Fig. 7 ***Conidiobolus coronatus*** Culture tube showing "cloud" of discharged conidia on the glass above the growing colony.

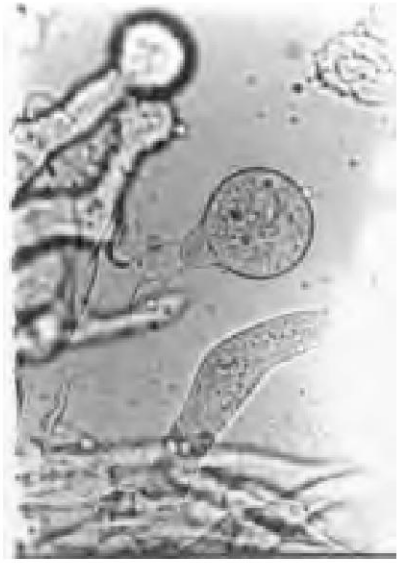

Fig. 8 ***Conidiobolus coronatus*** Conidiophores and mature conidium just prior to its violent discharge.

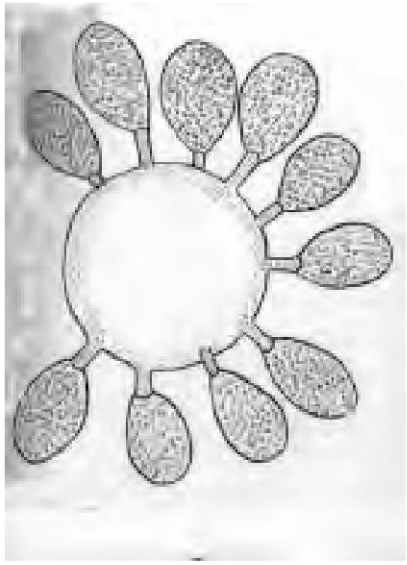

Fig. 9 ***Conidiobous coronatus*** Microconidia formation on radial sterigmata

Plate 5

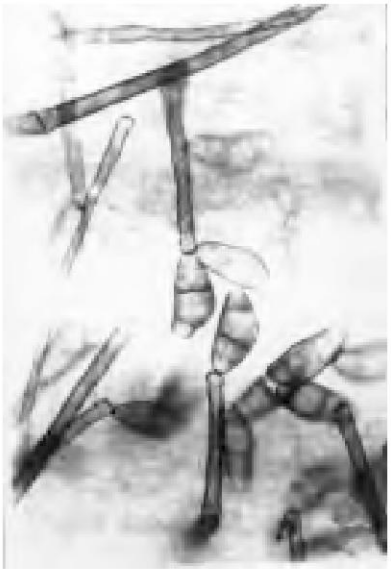

Fig. 13 ***Curvularia lunata***
Conidiophores and large multiseptate lunate conidia.

Fig. 14 ***Fusarium sp.***
Whitish mycelial colony on MGYP agar.

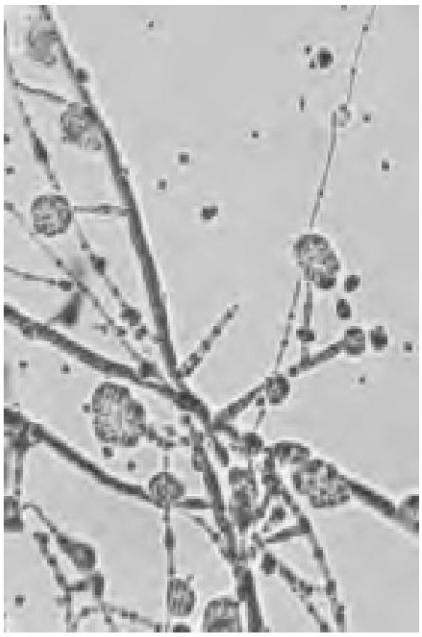

Fig. 15 ***Fusarium sp.***
Mycelium and conidia borne in slimy matrix on aerial mycelia.

+CMYK

Plate 6

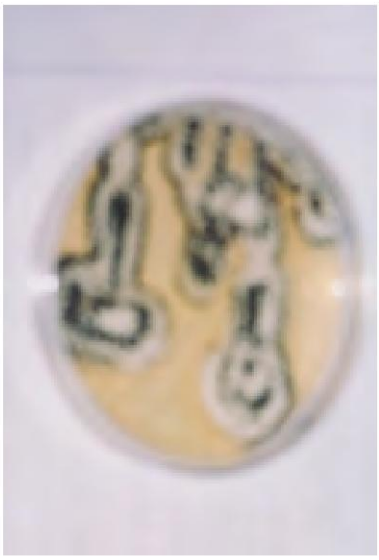

Fig. 16 ***Myrothecium verrucaria*** Colony growing on MGYP agar showing blackish masses of conidia borne in confluent sporodochia.

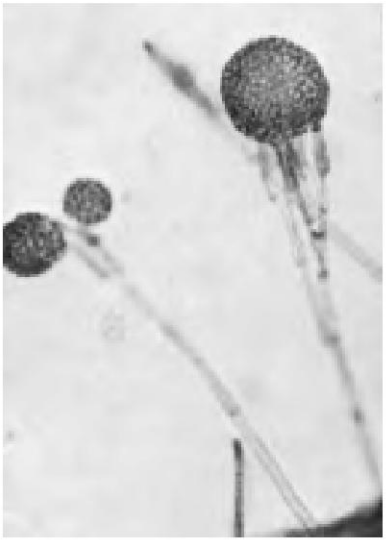

Fig. 17 ***Gliocladium sp.*** Conidiophores showing slimy masses of conidia.

Fig. 18 ***Gliocladium sp.*** Columnar chains of conidia (referable to *Clonostachys*).

+CMYK

Fig. 19 ***Penicillium sp.***
Conidiophores bearing chains of conidia.

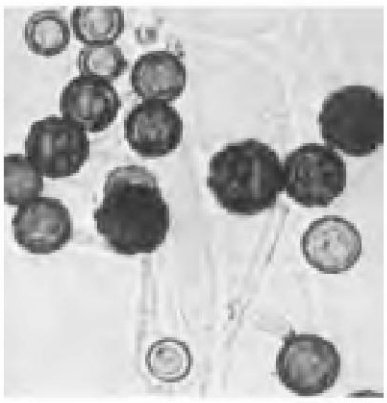

Fig. 20 ***Thermomyces (Humicola) lanuginosus***
Large single conidia with warty walls borne on short undifferentiated conidiophores.

Fig. 21 ***Trichoderma sp.***
Heavy sporulating colony showing greenish yellow masses of conidia.

Plate 8

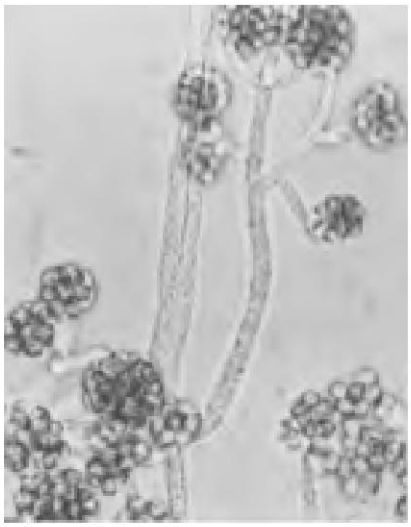

Fig. 22 ***Trichoderma sp.*** Conidiophores bearing conidia in slimy matrix.

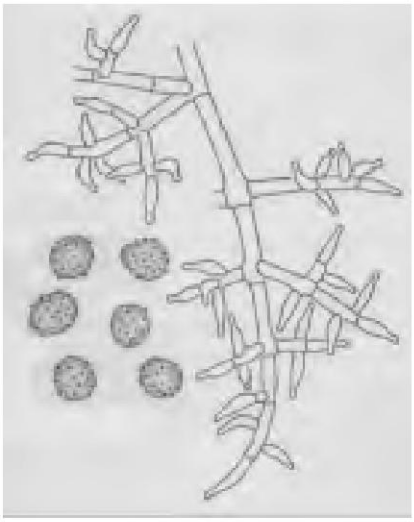

Fig. 23 ***Trichoderma viride*** Branching pattern of conidiophores and warty conidia.

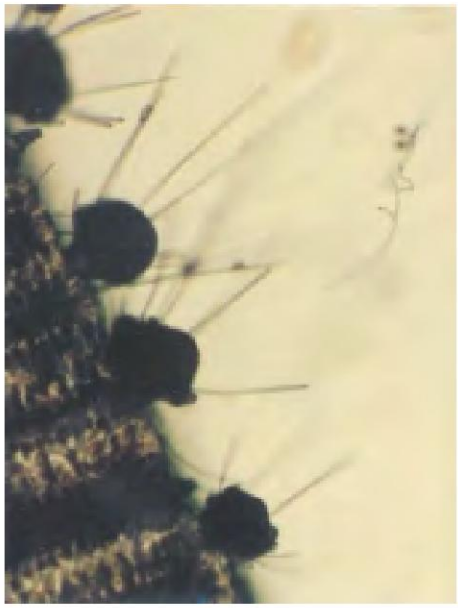

Fig. 24 ***Volutella sp.*** Sporodochia with subtending setae developing on sterile grass leaf.

Plate 4

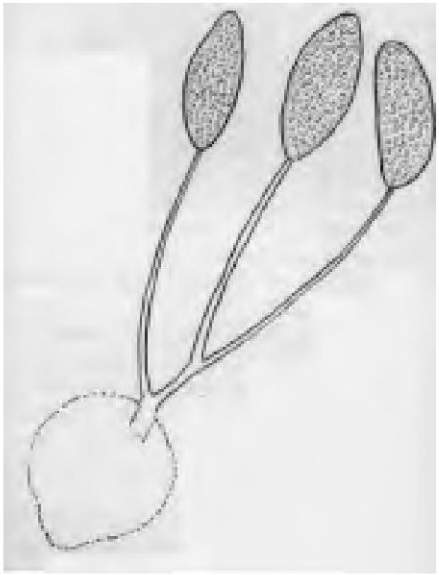

Fig. 10 ***Conidiobolus heterosporus***
Heteromorphic elongate conidia formation on branched slender conidiophores.

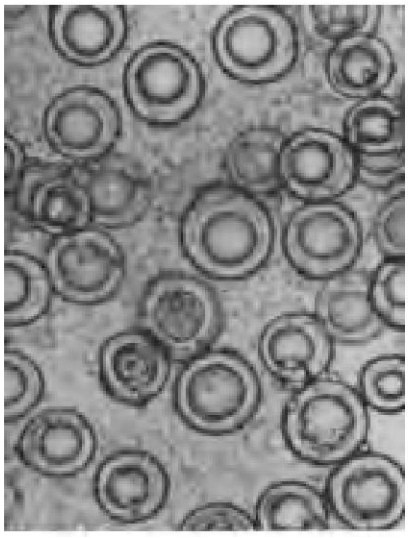

Fig. 11 ***Conidiobolus brefeldianus***
Mature zygospores with thick smooth walls and large reserve globule in the centre.

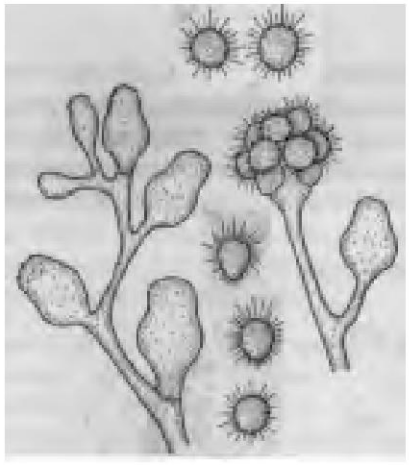

Fig. 12 ***Cunninghamella sp.***
Conidiophores and conidia with prominent spiny projections.

Fig. 25–28 ***Benjaminiella multispora*** (Dimorphic Mucorales)

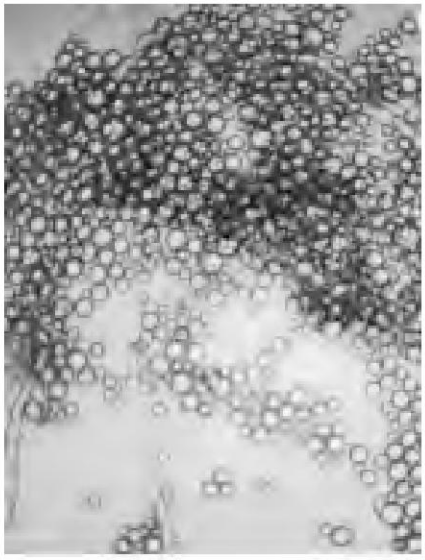

Fig. 25 "Yeast phase" cells settled at the bottom of MGYP broth cultures.

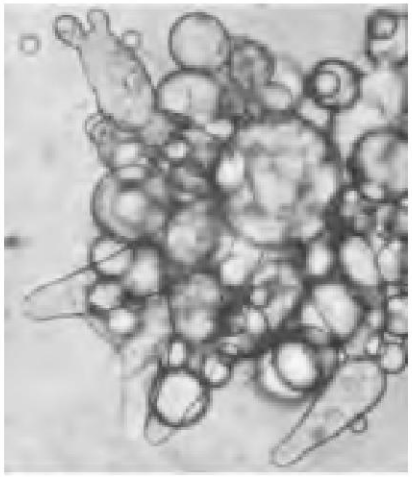

Fig. 26 Initiation of mycelial growth from yeast phase cells on agar media.

Fig. 27 Non-columellate multispored sporangioles.

Plate 10

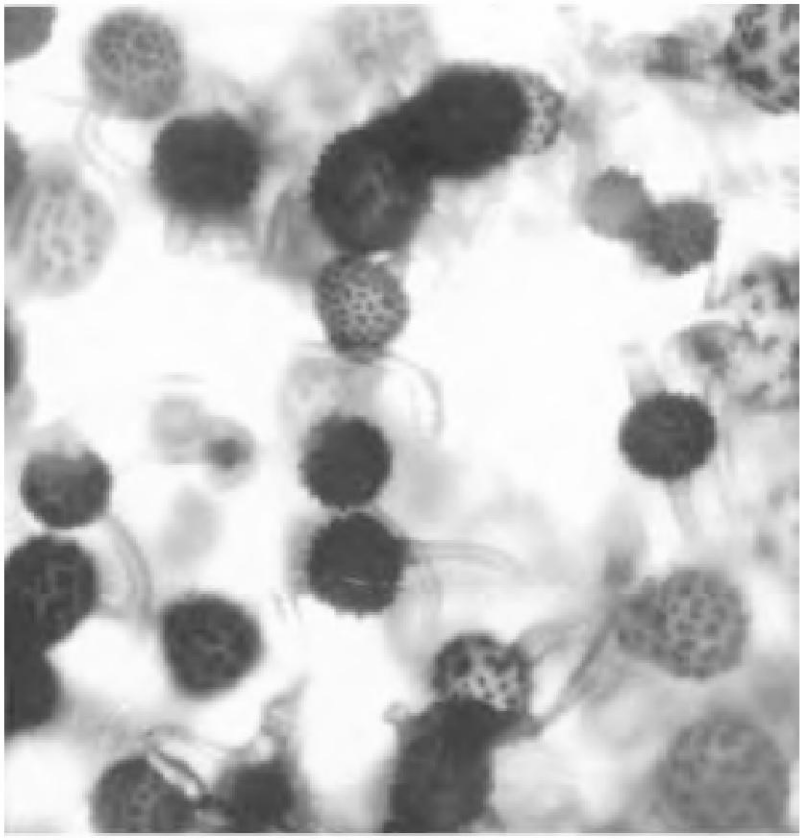

Fig. 28 Zygospores formed by conjugating gametangia on short aerial branches of mycelium.