MULTIPLE PROTEOLYTIC ENZYME PRODUCTION

IN KERATINOPHILIC FUNGI

(Preliminary Investigations)

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ABSTRACT

Superficial fungal infections can be acquired from a number of sources, e.g. animals, humans or from the soil. Many of the fungal species commonly associated with human disease arise from infection by species known commonly as dermatophytes, although infection from other non-dermatophytic keratinophilic fungi is becoming more common.

Other species not commonly regarded as pathogenic have on occasion been found in human infection. Many of these opportunistic species are commonly found in soils.

Isolation procedures employed in these studies were the hairbrush technique for small animals and the keratin-baiting technique for soil with samples being cultured on SDA containing antibiotics. Soil samples yielded 3 keratinophilic genera found in human infection (*Microsporum spp.. Trichophyton spp. Aphanoascus sp.*) while fungi isolated from animals yielded 3 fungal species, *Microsporum canis, Microsporum cookei* and *Scopulariopsis brevicaulis.*

In these studies, various culture parameters e.g. pH, spore numbers and various hydrolysis techniques were examined in order to asses the production of proteolytic enzymes *in vitro.* Also in the course of these studies, the use of lactrimel medium as a suitable recovery agent for strains presenting atypical colony morphology and reduced proteolytic enzyme production was trialed with excellent results.

The gelatin SDS-PAGE technique, mode of culture (shake and stationary) and the effect of substrate were analysed to compare the effects that these have on a range of keratinophilic fungi. Both pathogens and saprophytes were examined in an attempt to detect similarities in enzyme production which could be associated with the ability of various species to invade skin *in vivo.*

A large body of data has been gathered demonstrating that the proteolytic enzymes produced by most keratinophilic fungi encompass a wide range of MW sizes and are not entirely predictable. This strongly suggests that when these fungi come into contact with a particular substrate, the ability of the strain to adapt may depend on the strains ability to produce a proteolytic enzyme capable of breaking down the substrates in the external environment providing nutrients for the growing fungi.

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INTRODUCTION

1.1 Keratinophilic Fungi

Fungi are an integral part of the biomass of any natural environment, including soils, where many species have become specialised in the evolutionary processes to invade, colonise and thereby ensure decomposition of keratinous debris of other organisms (Ajello, 1974). These fungi utilise subsequent protein products as nutrient sources for continued growth and reproduction. They can be termed keratinophilic or keratinolytic, with their lytic abilities attributed in varying degrees to the production of various structures enabling physical invasion of substrates (Davidson & Gregory, 1934, Vanbreuseghem, 1949,1950,1952, English, 1963, Barlow & Chattaway, 1955, etc.) together with the production and secretion of various proteases, lipases and keratinases (Yu *et al.*, 1968, 1969).

The ability to colonise natural keratin is widespread and a considerable number of species within the aquatic fungi (*Chrytridiales, Saprolegniales),* zygomycetes, ascomycetes and deuteromycetes are reported with this attribute. These fungi have been placed into two different groups: 1) dermatophytes and other species able to attack native keratin by penetration (Vanbreuseghem, 1952, English , 1963) as well as enzyme activity (Vanbreuseghem,1953, Mathison, 1964) and 2) saprophytic species which attack the keratin mainly by hyphal penetration to various extents (English, 1963) or simply surface growth, utilising the more easily decomposable compounds of the keratin substrate (English, 1965, Apinis, 1967).

In general, keratinous materials which fall to the ground are successively colonised by differing groups of fungi. The *Chrytidiaceae*, which can be seen microscopically only, may colonise first, followed by non-keratinolytic species, often with representatives from the genera *Fusarium, Penicillium, Mucor & Diheterospora.*

These fungi assimilate the easier to digest substances which are probably present in the intercellular substance of the substrate. A third group is able to break down the more resistant substances found there e.g. *Chaetomium, Gliocladium, Humicola & Scopulariopsis brevicaulis,* while the final group is formed by true keratinolytic species such as *Microsporum, Trichophyton & Chrysosporium spp.* (DeVries, 1962).

Several genera of these fungi include species which are pathogenic to man and other animals. The most important are species of the 'dermatophytes', *Microsporum, Trichophyton & Epidermophyton,* which are able to actively invade the keratinised tissues resulting in 'dermatophytoses', but invasion by other keratinophilic species is not uncommon and such infections are frequently referred to as opportunistic.

1.2 Identification and Classification of Keratinophilic Fungi

Identification of the various species has in the past been based on traditional methods of observation of both gross colony morphology on Sabouraud's Dextrose Agar (SDA) and microscopic examination of spores.

Important colony characteristics for the dermatophytes and other species include rate of growth, topography, texture and reverse and surface pigmentation. As colony appearance and pigmentation depends on the medium used for culture, SDA is used to obtain colonies which may be compared to others reported in the literature (Ajello, 1966).

Also commonly used for dermatophytes is dermatophyte test medium (DTM). This is a selective medium which excludes most bacteria by the incorporation of chloramphenicol, which inhibits the peptidyl transferase activity of the 50S ribosomal subunit in prokaryotes, and also inhibits non-dermatophyte fungi via the use of cycloheximide which blocks the peptidyl transferase activity of the 60S ribosomal subunit in eukaryotes (Stryer, 1995). No sensitivity to this latter has been found among dermatophytes. This medium also contains a yellow - red pH indicator rapidly affected by dermatophytes and related species, allowing early recognition (Rebel &Taplin, 1970).

Other methods employed in identification include the use of selective media based on known physiological characteristics of a particular strain, such as partial or essential nutrient requirements, e.g. *T. equinum* -niacin or *T. meginii* - l-histidine, (Geog *et al.*, 1957) and *T. tonsurans* var. *sulfureum* - thiamine (Padhye *et al.*, 1994).

Microscopic examination of various species shows that most dermatophytes have two types of conidia/aleuriospores: multicelled macrospores (macroaleuriospores) and small unicellular microspores (microaleuriospores).

Macrospores of the genus *Microsporum* tend to have thick, rough walls, while those of *Trichophyton* and *Epidermophyton* are thin, smooth walled, although various strains of *Microsporum spp.* have been known to produce smooth walled macrospores (Rebel & Taplin, 1970).

Microspores are produced by most parasitic dermatophytes on occasion, except for *Epidermophyton floccosum* which is unique in that it does not produce microspores (Rebel & Taplin, 1970).

There is also a range of vegetative structures seen microscopically which can be useful in identifying various strains or species, i.e. chlamydospores, spiral hyphae, antler-shaped hyphae (chandeliers), nodular organs and pycnidia-like structures (Emmons, 1934, Ajello, 1966, Rebel & Taplin, 1970).

Identification criteria of other keratinophilic species are based on gross and microscopic characteristics listed in various publications e.g. for *Chrysosporium & Aphanoascus* -Carmichael (1962), Apinis (1967), Barron (1968), Rippon *et al* (1970) Cano & Guarro (1990), for *Scopulariopsis brevicaulis* - Barron (1968), Gravesen *et al.* (1994), for *Diheterospora* and it's *Verticillium* states - Barron (1968,1985).

1.2.1 The Teleomorphic States

As the perfect or teleomorphic states of a number of the dermatophytic and other species have been discovered, changes have been made to classifications (Ajello, 1974,1977, Vanbreuseghem, 1977, Takashio, 1979). For example, the perfect states of *Microsporum spp.* have been named as *Nannizia spp.* and the perfect states of *Trichophyton spp.* named as *Arthroderma sp.* (Table 1.1)

The teleomorph genus *Aphanoascus* (anamorph *Chrysosporium spp.*)has suffered taxonomic confusion since its foundation by Zukal (1890). It was extensively reviewed by Apinis (1967), who considered *A. fulvescens* (Cooke) Apinis the type species. The species *A. terreus* was originally identified by Randhawa & Sandhu (1964) who named it *Keratinomyces terreus*, it was later renamed following comparison with a culture from Centraalbureau voor Schimmelcultures, Baarn (Apinis, 1967).

Subsequent mating studies have also revealed that some of the anamorphic states represent a complex of species, e.g. the *M. gypseum-fulvum* complex and the *T. terrestre* complex.

The biological group of dermatophytes and some other fungi e.g. *Ctenomyces serratus*, a species with a strong predilection for feathers, are now classified in the family Arthrodermataceae and it is in this group that keratinophily or a predilection for keratin substrates is fully expressed (Leclerc *et al.*, 1994).

Table 1.1: The major *Trichophyton & Microsporum* species with known teleomorphic states.

Teleomorph	Anamorph
Trichophyton spp..	
Arthroderma Berkley, 1860	*Trichophyton* Malmsten, 1845
A. benhamiae Ajello & Cheng 1967b	*T. mentagrophytes var mentagrophytes*
A. insingulare Padhye & Carmicheal, 1972	*T. terrestre*
A. lenticularum Pore, Tsao & Plunkett, 1965	*T. terrestre*
A. quadrifidum Dawson & Gentiles, 1961	*T. terrestre*
A. uncinatum Dawson & Gentiles, 1961	*T. ajelloi*
A. vanbreuseghemii Takashio, 1973	*T. mentagrophytes var. interdigitale*
Microsporum spp.	
Nannizzia Stockdale, 1961	*Microsporum* Gruby, 1843
N. cajetani Ajello, 1961	*M. cookei*
N. grubyia Georg & Ajello Friedman & Brinkman, 1962	*M. vanbreuseghemii*
N.gypsea Stockdale, 1963	*M. gypseum*
N. incurvata Stockdale, 1961	*M. gypseum*
N. obtusa Dawson & Gentiles, 1961	*M. nanum*
N. otae Hasegawa & Usui, 1975	*M. canis*

Modified from Ajello (1968, 1974), Vanbreuseghem *et al.* (1977), Howard (1983) and Simpanya (1994).

1.2.2 Molecular Mycology in Identification Processes

Although fungal taxonomy relies upon traditional morphological examination, several chemotaxonomic methods have been applied to dermatophytes, including fatty acid analysis (Jones & Noble, 1981) and sterol composition (Jones & Mallet, 1983) with the latter method tending to show division of species according to ecological origin. However, clinical mycology laboratories are increasingly utilising molecular based methods for the recognition of pathogenic fungi involved in human disease. Central to this is the design and use of DNA/RNA probes and primers for use with the polymerase chain reaction (PCR).

The basis for investigations of this type is that DNA sequences can be found which are common to all fungi but differ to those found in non-fungal species, in particular humans, eukaryotic parasites and bacteria, thereby addressing the question "Is the infection fungal rather than bacterial?" (Mitchel *et al.*, 1994).

DNA homology studies of dermatophytes tend to support the present classification system (Davidson *et al.*, 1984, Taylor, 1986) although there are still some ambiguous results e.g. unexpected low homologies between anamorphs and teleomorphs. However, using mitochondrial DNA (mtDNA) from the sexual and asexual states, Kawasaki *et al.* (1995, 1996) concluded that there was no distinction between the genera *Arthroderma & Nannizzia*, which supports the work of earlier researchers (Weitzman *et al.*, 1986).

Recently, Cano *et al.* (1996) have felt that they have had some success with using mtDNA Restriction Fragment Length Polymorphism (RFLP) techniques in classifying various strains of *Chrysosporium sp.*, achieved using restriction enzymes with G - C four base recognition. However, they have stated that the phylogenetic value of this technique is limited, due to the numbers of mtDNA able to be studied being few in number.

It is believed that the ribosomal RNA gene (rDNA) region in fungi contains areas with enough sequence conservation for primers and probes at relatively deep taxonomic levels, and other areas with enough variability for some species-specific discrimination. Being a multi copy gene it may be considered a "pre-amplified" target, with further amplification increasing the sensitivity for detecting organisms few in number in a clinical specimen (Mitchel *et al.*, 1994).

Sequence comparisons of various subunits of the rDNA region is enabling researchers to investigate phylogeny of dermatophytes and other fungi, as well as investigate approaches to the detection and identification of pathogenic fungi (Olsen *et al.*, 1986, Leclerc *et al.*, 1994, Harmsen *et al.*, 1995, Haynes *et al.*, 1995).

Although at present there are no well developed probes available for the detection of commonly found dermatophytes, currently available acridinium ester-labelled DNA probes directed against rRNA have been evaluated for other fungi such as *Histoplasma capsulatum* and *Cryptococcus. neoformans*, with specificity and sensitivity for these fungi near 100% (Benek & Rogers, 1996).

1.3 Ecology

It was initially hypothesised in 1893 and 1910 by Sabouraud that dermatophytes live as saprophytes in soil during a part of their life cycle. This hypothesis was taken up by Davidson & Gregory (1932,1934) who assumed that dermatophytes grew on keratinaceous debris in the soil, and the term 'keratinophilic' was later applied to all pathogenic fungal species, not only dermatophytic, which could be isolated on keratin baits (de Vries, 1962).

The use of specialised isolation techniques for the study of keratinophilic fungi was introduced by Karling (1946) for his study of keratinophilic *Chrydiaceae.* He grew these fungi on hairs or skin scales which he had put on the surface of the soil or substrate to be investigated. This technique was used extensively by Vanbreuseghem (1952), and is now commonly known as the "Hair Baiting Technique".

Soil samples being investigated for keratinophilic fungi are usually taken from the surface layer of soils and placed into suitable receptacles. Once back in the laboratory, the soil is placed in a sterile petri dish, moistened with sterile distilled water and "baited" with pieces of sterile keratin such as feathers, wool or hair. The sample is then incubated at 25°C and examined daily for fungal growth (Plate 1.1).

A large number of pathogenic and potentially pathogenic fungi from a range of genera have been isolated from soils in all parts of the world and the presence of an abundant keratinophilic fungal flora in soils has been revealed (Ajello, 1956,1959, Marples, 1965, Chmel *et al.*, 1972, Lee, 1979, Mercatini *et al.*, 1993, Simpanya *et al.*, 1996, Currah *et al.*,1996). Thus the soil is a very important environmental reservoir of pathogenic fungi.

Infections caused by some of these species involves an unlimited host range and demonstrates the wide ranging versatilities of some fungi. But among the dermatophyte group there is a host range in which individual species differ, with the differences in host specificity considered attributable to the molecular differences in keratins of various hosts (Rippon, 1982).

With the probability that some fungal pathogens have evolved from an original natural habitat in the soil and then developed host specificity, dermatophytes were divided by Georg (1959) into three ecological groups:- geophiles, zoophiles and anthropophiles.

Plate 1.1 Soil Plate "Keratin Baited" with Sheep Wool

Fungal growth clearly visible on wool samples

1.3.1 Geophilic Fungi

Keratinophilic fungi in this group occur as saprophytes in soils, particularly those rich in keratinous substrates. Their presence in the soil is determined by a number of factors e.g. pH , temperature and availability of keratin, which influence their ability to grow and survive. A few of these species under suitable conditions have been known to cause dermatophyte infection in animals and humans (Woodgyer, 1994). Although the lesions caused during these infections may be severe, they usually heal spontaneously.

The more pathogenic strains of geophilic dermatophytes are sometimes found to show characteristic traits of true pathogens e.g. production of arthrospores in invaded skin. The principle virulent geophilic dermatophyte is considered to be *M. gypseum*.

This species has been well documented world-wide as a pathogen of man and animals, with infective propagules originating from saprobic sources transmitted directly or indirectly to the host (Ajello *et al.*, 1966, 1974, De Vroey, 1984,1985, Woodgyer, 1994). More recently, the *M. gypseum* has come to light as a complication in immunocompromised patients such as those with acquired immunodeficiency syndrome, with clinical manifestations being more severe and with a wider variation than those found in non-immunocompromised patients (Porro *et al.*, 1997).

Other species which have been rarely associated with human infection are *M. cookei* (Frey, 1971) and *T. ajelloi* (Presbury & Young, 1978). In addition, infections from non-dermatophytic geophilic fungi or opportunist infections, occurring either singly or as part of a mixed infection, have been recorded with e.g. *S. brevicaulis* and *Chrysosporium sp.*,(Woodgyer, 1994, 1995,1996., Velez *et al.*, 1997) and an infection from an *Aphanoascus sp.* has been recorded (Rippon *et al.*, 1970).

Currently, there are no reported cases of human infection from members of *Diheterospora sp.*

1.3.2 Zoophilic Fungi

Zoophilic fungi are mainly animal pathogens but several are also able to infect humans. The principal dermatophytes in this group are listed in Table 1.2 (Woodgyer, 1994).

Table 1.2 Common zoophilic dermatophyte species in NZ and most common animal host.

Dermatophyte	Cat	Dog	Rodent	Guinea Pigs	Rabbits	Hedgehogs	Cattle	Horses	Pigs
Microsporum canis	F	IF			R			IF*	
M. equinum								R**	
M. nanum									F
Trichophyton equinum (both varieties)		R***					R	F	
T. mentagrophytes var erinacei	R	IF				F			
T.mentagrophytes var mentagrophytes	R	R	F	F	R	R	R	R	
T.verrucosum							F	R	

legend: F = Frequently isolated
IF = Infrequently isolated
R = Rarely isolated

*** Identified as *M. canis* but possibly *M. equinum***
**** Rare cause of equine ringworm in North Island**
***** Case diagnosed where dog had contact with infected horse**

Humans, particularly children in the age range 5 - 14 yrs, readily acquire infections through direct contact with infected animals or via infected fur and hair shed by domestic pets, particularly kittens and puppies. Infections with one of the above species are highly inflammatory and tend to be self-limiting. Treatment with antifungals in these cases tips the balance in favour of the host and the majority of infections respond favourably to such treatment.

The two most common agents of human infection from the above group of fungi are *M. canis*, which is a frequent cause of tinea capitis in pre-pubertal children accounting for up to 88% of culture diagnosed cases (Woodgyer, 1993) and *T. mentagrophytes*. Although *M. canis* is not found in the soil, *T. mentagrophytes* has occasionally been isolated from soil (Baxter, 1966, Padhye & Carmicheal, 1968) where it can survive for several months.

1.3.3 Anthropophilic Fungi

Anthropophilic species are primarily adapted for parasitism of man, with the 3 main genera involved in human infections being *Epidermophyton, Trichophyton & Microsporum spp.*. These do not form part of the natural fauna of the hair and skin and although humans are the primary host, there have been occasional reports of infections in animals (Kaplan *et al.*, 1957, Georg, 1960). An outstanding feature of these dermatophytes is the large number of species specifically adapted to parasitize humans, larger than the total number of species adapted to parasitize all other mammalia (Tanaka *et al.*, 1992).

These species are commonly associated with community living and transmission is from host to host via direct contact, or by indirect contact via the shedding of infectious propagules into communal areas such as swimming pools, changing rooms, barracks and dormitories. In closed communities, the use of common personal items can lead to the rapid spread of infection throughout that community.

T. rubrum is an extremely common cause of skin infections world wide, affecting all populations and ethnic groups (Rippon, 1985, De Vroey, 1985). It is becoming increasingly more prevalent in urban populations, particularly with populations that commonly wear tight fitting shoes and clothing, which maintain heat and humidity (Philpot, 1977).

T. tonsurans is another species which is emerging as a very common cause of tinea capitis in New Zealand, but due to its highly variable manifestations, infections are frequently misdiagnosed as other dermatologic disorders such as seborrhoea, dandruff and impetigo (Woodgyer, 1993).

Anthropophilic infections tend to produce less inflammatory and more chronic disease states, suggesting that these species are very well adapted to the human host, particularly by comparison to infections caused by most zoophilic or geophilic species (Sohnle, 1989). This aspect can make detection or correct diagnosis difficult and cases of "tinea incognito" are not unknown, with various patients being diagnosed as having bacterial infections. In two such cases the infectious agent was in fact found to be *T. verrucosum* and in others the agent has been found to be *T. tonsurans* (Woodgyer, 1993, 1994).

1.4 Keratin

Keratins are a major constituent of cells and tissues largely unreactive toward the environment and mechanically quite strong (Fraser *et al.*, 1973, Mercer, 1961) but also are substrates which dermatophytes and other keratinophilic fungi are able to utilise as a nutrient source.

They are structural proteins forming cytoplasmic intermediate filaments (IF) in cells such as those found in the epidermis. Keratin is not a single material but a complex of sulphur-containing proteins with a large number of cysteine residues initially existing in the thiol form but, during the keratinization process, these thiol groups are oxidised in pairs leading to the formation of diamino acid cysteine or disulphide bonds. It is thought these linkages are responsible for the insolubility of keratinized tissue and its resistance to enzymatic proteolysis. It is these linkages which are thought to be attacked by secreted proteases of keratinophilic fungi.

Other cytoplasmic intermediate filaments in vertebrate cells are vimentin and vimentin related filaments and neurofilaments. (Alberts *et al.*, 1994). In each type of IF the protein monomers involved are all highly elongated fibrous molecules having an amino-terminal head, a carboxy-terminal tail and a central rod domain. The central rod domain consists of an extended α-helical motif called the heptad repeat. This seven amino acid sequence motif promotes the formation of coiled-coil dimers between two parallel helices.

Although of common embryological origin and sharing a common structure, the keratins cover a very large range of amino acid compositions (Gillespie, 1990), and can be subdivided into two types: acidic (Type I) and neutral/basic(Type II) keratins. Keratin filaments are heteropolymers formed from equal numbers of type I and type II keratin polypeptides (Alberts *et al.*, 1994).

Keratins are commonly divided into three groups on the basis of the structures adopted by their various protein constituents rather than on their physical attributes. These are a) α-keratins which include "Hard" keratins such as hair, nails, claws etc. and the "Soft" epidermal keratins which form the stratum corneum, corns and callouses, b) β-keratins which are not naturally occurring but which are produced by the action of pressure and temperature on native α-keratins and c) "Feather" keratins, which include feathers, scales and also parts of beaks and claws (Parry, 1996).

1.4.1 Hard and Epidermal Keratin

X-ray diffraction patterns of hard and epidermal α-keratin have been interpreted in terms of filamentous assemblies of highly orientated molecules with conformations based on the right-handed α-helix.

Hard α-keratin IF are embedded in matrix proteins. The matrix consist of three families of proteins: those rich in cysteine residues (the high-sulphur proteins), those extremely rich in cysteine residues (the ultra high-sulphur proteins), and those rich in glycine and tyrosine residues (the high tyrosine proteins). The content and composition of the matrix proteins vary with source, age and nutrition (Parry, 1996, Fraser *et al.*, 1973, Mercer, 1961).

Although the matrix proteins as a group play an important role mechanically, especially in compression, it is the number and disposition of the covalent disuphide bonds formed within and between the matrix proteins and the terminal domains of the IF molecules that dominate the physical attributes of hard α-keratin.

There is no matrix as such in the epidermal keratins, but IF associated proteins (IFAP) such as filaggrin are responsible for aggregating IF into larger assembles that act as the functional unit *in vivo*.

1.4.2 β -Keratin

This particular form of keratin does not occur naturally but can be readily produced when mammalian hard α-keratin is stretched by about 100% in steam over the course of five to six hours. The specimen must then be held in this extended state in the presence of steam for a couple more hours in order to set the structure in the β form, and hence prevent its conversion back to the α-structure. This setting mechanism, in a modified and less severe form, is commonly used in the clothing industry and in hairdressing to maintain shape and form.

β-Keratin provides a neat structural link between the α-keratins and the feather structure. It has also provided insight into the relationship between the chain and molecular structure of epidermal keratin and its filamentous aggregate (IF) (Parry, 1996).

1.4.3 Feather and Reptilian Scale Keratin

The hard keratin in avian epidermal appendages such as feathers, beaks, and claws can largely be accounted for by a single protein species with a molecular weight of about 10.4 Kilodaltons (Kda). While this feature is dissimilar to that seen in the α -keratins, where distinct families of filament-forming and matrix proteins were identified, electron microscopy has shown that feather keratin also consists of filaments embedded in a "matrix", with filaments only about 3.3 nm in diameter. This has been confirmed by X-ray diffraction patterns. Feather keratin protein is unique, however, in that it forms both the filament and the "matrix".

The amino acid sequences of feather and scale keratin display a high degree of homology. But scale keratin molecules have a much higher molecular weight than those of feather keratin (15 Kda vs 10.4 Kda) and a much higher degree of lateral order.

The homology between feather and scale keratin implies that these two proteins have a common ancestor. Since scales occurred well before feathers in an evolutionary time scale, it is likely that feathers evolved from scales as a result of the deletion of the 4 x 13 residue motif found in scales but not in feathers (Parry, 1996).

1.4.4 Physical Properties of Keratins

Each of the keratinous tissues has unique features that enable it to function optimally, for example, the thermal insulation attributes of hair necessitate that it must be flexible and moderately extensible. In addition, water sorption properties are important. In contrast, it is easily seen that the hard α-keratin of rhinoceros horn (which is actually hair) and the epidermal keratin that forms the surface layer of skin must have quite different physical attributes. In contrast yet again, feather keratin must be light and inextensible without being brittle.

The hard α-keratins (but not the soft ones) both have a filament-matrix texture. The 10 nm -diameter intermediate filaments (IF) are of high elastic modulus and provide axial strength, and the matrix of low elastic modulus resists compression. This mixture allows stress in the tissue to be distributed uniformly and, in addition, provides a natural crack-resist mechanism.

The physical properties of keratins do not depend solely on the composite nature of the material. Other aspects that are important include the water content, the lipid content (particularly in epidermal keratins) and the composition and content of the proteins that constitute either the matrix or the proteins associated with the IF (the IFAP).

The highly disulphide-bonded structure of the α-keratins present a firm barrier to the environment, in contrast to the flexible barrier presented by the epidermal keratins. Superficially at least, the IF in the epidermis are similar to those in the hard α-keratins. But the N- and the C- terminal domains of the constituent molecules have very different characteristics, leading to the differing structures with differing physical attributes. The cysteines in hard α-keratins will form disulphide bonds to give rigid and tough structures whereas the glycine and serine residues in epidermal keratin will form many weak van der Waals interactions with other IF molecules and IFAP. The matrix of the hard α-keratins is absent in the epidermal keratins, affecting the properties of the latter and leading to a more flexible ultrastructure.

Orientation of the IF in the aggregates that form are also crucial to the function of a particular cell. For example, in wool and hair, they lie parallel to the axis of the fibre, yet in tactile whiskers a large fraction is tangentially disposed. In human nail the IF lie parallel to the surface but are perpendicular to the direction of growth, whereas in stratum corneum, the bundles of IF, which are roughly parallel to the skin surface, are woven into a fabric-like structure. Therefore orientation can play a leading role in specifying mechanical properties.

The natural resistance of keratin fibres to degradation by chemicals or enzymes is well known and related to their protective function in nature. It is the heterogeneous nature of keratins and the varying compositions of non-keratinous components which governs both the specificity of certain fungi to particular types of keratinous substrates and to the growth-pattern adopted. Furthermore, the structure of the keratin can be markedly altered by pre-treatment such as autoclaving e.g. hair and wool are richer in the "tougher" keratins than nail, hooves, horns and the stratum corneum of the skin.

All these factors make the choice of substrate to be used in studies of the legitimate breakdown of keratin difficult.

1.5 Colonisation of Keratins by Fungi

One of the distinctive properties of keratinophilic fungi is the ability to colonise and to breakdown keratin and there have been many investigations concerning the mechanisms involved. The colonisation process itself is a dynamic one with substrate, environment and fungal factors involved. This holds true where invasion involves a dermatophyte or a non-dermatophyte opportunist propagule. (Raubitschek, 1961, Kunert, 1972, Minocha *et al.*, 1972, Ruffin *et al.*, 1976, Meevootisom *et al.*, 1979).

During the initial phase of colonisation a propagule must make contact with a surface and adherence must occur in order for germination, colonisation and invasion to proceed. *In vitro* studies by Zurita *et al.*,(1987) showed that adherence of both saprophytic macro and microspores and parasitic arthrospores to human keratinocytes occurs in a time dependant fashion, with maximum adherence of both microspores and arthrospores occurring within 3 - 4 hours.

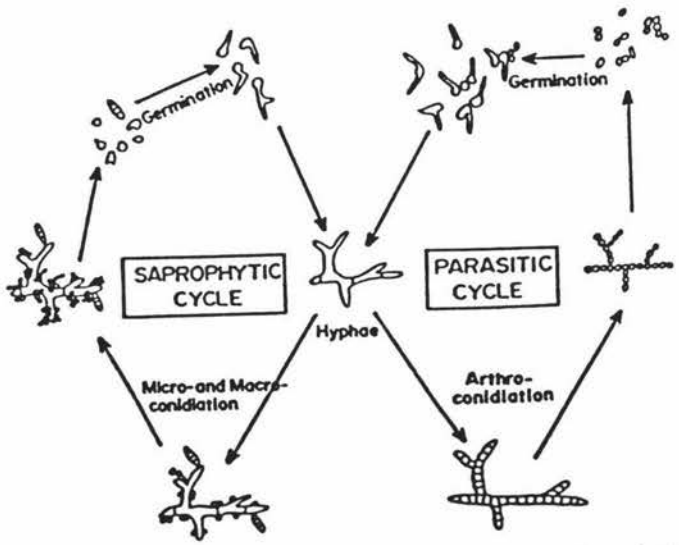
The adherence mechanism is not well understood due to a lack of satisfactory models with which to study early colonisation events (Bhattacharya *et al.*, 1998). However, it is known that adherence is enhanced by factors such as hydration, with water considered a suitable vehicle for the transfer of a propagule from host to host.

Work done by Tsuboi *et al.*,(1994) showed that with *T. mentagrophytes*, adherence appeared mediated by germling outer cell fibrils while further work by Ogawa *et al.*, (1998) demonstrated very close contact between dermatophyte arthroconidia and corneocytes in ultrastructural studies. Where contact is made, a fibrillar-floccular material, the nature and origin of which is yet to be determined, is seen in the space between the arthroconidial outer wall and the corneocyte membrane.

The above findings suggest that germination itself may be a virulence factor involved in disease initiation, particularly as a spore which fails to germinate will be removed by the normal and continuous desquamation of the epithelium. (Tsuboi *et al.*, 1994, Ogawa *et al.*, 1998).

In vivo dermatophyte infection usually follows indirect or direct contact with infected scales or hairs containing arthrospores and the chances of successful transmission is considerably improved by the prolonged viability of these arthrospores in exfoliated skin (Dvorak *et al.*, 1968, Sohnle, 1989). Successful invasion is followed by proliferation of the organisms with the extension of the infection into the surrounding area and at times from one structure to another e.g. skin to hair or nail.

Depending on the environment in which the fungus grows, one of two types of asexual propagule can be produced, the saprophytic and parasitic spores/conidia. Under non-parasitic conditions often two distinct spores are seen, saprophytic macrospores and microspores, both formed in the holothallic mode by conversion of a terminal or intercalary segment of a fertile hypha into a propagule surrounded by a multilayered wall. The parasitic arthrospores however, are formed in the thallic-arthric mode by consecutive segmentation and fragmentation of an existing hypha (Cole & Samson, 1979).



The asexual life cycle of the dermatophyte *T. mentagrophytes*. When fertile hyphae enter the saprophytic cycle, they proliferate by apical elongation and lateral branching forming macro- or microconidiogenous hyphae. Swelling of the apical tips of the conidiogenous hyphae is the initial event that leads to macro- or microconidium formation. Under parasitic conditions, fertile hyphae are repeatedly segmented and eventually fragmented to form arthroconidia.

Fig 1.1 Asexual cycle of *T. mentagrophytes.*

Fig by Emaynitoff and Hashimoto (Cole & Hoch, 1991)

The regulatory elements of development of one state in favour of another are largely unknown. However, it is recognised that the mechanism controlling saprophytic sporogenesis is suppressed during states of active invasion or parasitization of a host, with the production of parasitic arthrospores being the only asexual propagules formed in these circumstances (Cole & Hoch, 1991). It is notable, that while in theory infection can arise from the initial germination of either a saprophytic or parasitic propagule on a suitable host, it is commonly held that the arthrospore form is the major mediator responsible for disease transmission by anthropophilic dermatophytes.

However, infections caused by geophilic dermatophytes or by opportunistic non-dermatophytic keratinophilic fungi involves the uptake of a "saprophytic" propagule, e.g. infections caused by *M. gypseum*, a geophile, would almost certainly arise from the continued growth of a segment of vegetative hypha or the germination of a conidium on a suitable host. Although growth in the case of dermatophytes will be in the arthrospore form, in the case of opportunistic infections e.g. by *S. brevicaulis*, microscopic examination of samples directly from the lesion may reveal conidia, indicating that an "arthrospore" (or hyphal-yeast morphology such as that found in *Malasezzia furfur* and *Candida spp.*.) is not always required for fungal invasion.

Exposure to and uptake of an infectious fungal propagule by human or animal skin *in vivo* does not always result in infection and should infection occur, it may heal spontaneously or persist as a chronic infection. The type and severity of any given infection will depend upon the relationship occurring between the invading fungal species and host. The length and degree of infection is dependent upon the characteristics of the invading organism, the site of infection and both the innate and aquired components of the host immunological system (Sohnle, 1989, Ogawa *et al.*, 1998).

The stratum corneum itself *in vivo* represents an effective barrier to invasion by a fungus, with epidermal cell proliferation and keratinization processes presenting an important initial defence mechanism against infection. Furthermore, keratinocytes in the periphery of annular dermatophytosis lesions have been found to have an increased rate of cell division (Sohnle, 1989, Ogawa *et al.*, 1998). On the other hand, if infection has occurred the stratum corneum, due to its physical structure, may protect the invading organism from direct contact with effector cells of the immune system.

The major infiltrates of the cell-mediated immune response are generally confined to the dermis but some neutrophils and lymphocytes may enter the epidermis and are able to directly attack and kill pathogens by a variety of mechanisms e.g. the production of the antimicrobial protein, calprotectin which can inhibit fungal growth by competing with the microbes for zinc (Ogawa *et al.*, 1998).

Also found superficially are substances such as lipids containing saturated fatty acids that are active against organisms causing tinea capitis and others. More recently, the sphingosines have been characterized as an antimicrobial barrier in the skin (Ogawa *et al.*, 1998).

The invading organism must be able to obtain nutrients required for growth and the variety of proteinases, including keratinases, produced by dermatophytes, for example, facilitate penetration of the cornified tissues. In response to this, host proteins such as transferrin and complement contribute to resistance by restricting or inhibiting the growth of the organisms.

Cell-mediated responses can be an important line of defence with a number of cell types with immunological potential in the skin e.g. epidermal Langerhan's cells, dermal dendritic cells and epidermal T-lymphocytes, with these cells thought to interact with each other by means of surface markers, adhesion molecules, cytokines and eicosanoids to generate an inflammatory response. All of these aid in the development of resistance to infection.

However, defects in T-cell mediated immunity or in phagocytosis have been found to be important with fungal infection of all types, including dermatophytoses. Disease is more frequent and severe in patients with immunological defects (Ogawa *et al.*, 1998). Any number of systemic factors have been associated with decreased effectiveness of certain immunological functions, many of which are inheritable (Sohnle, 1989). Important also is the increasing incidence within the worldwide human population of AIDs. This condition involves the compromising of the immune system leaving victims vulnerable to secondary infection, a large number of which are caused by fungal agents, including superficial fungi (Porro *et al.*, 1997).

Overall, while deep infections with dermatophytes and opportunistic fungi do occurr, such infections are rare. Skin *in vivo* is a very effective barrier to invasion by these organisms with this barrier function consisting of a variety of components which provides an overall defence system, with the dual role of confining invading fungi to superficial epidermal sites and then eliminating them from the skin. Thus the body surface offers a variety of restraints on fungal growth *in vivo* which would not be operating *in vitro.*

The specialised category of anthropophilic dermatophytes, however, are particularly well adapted to the locations they parasitize as well as utilising keratins and other structural proteins as nutrient sources. They fulfill the requirements of a successful pathogen i.e. they have the ability to adhere to the stratum corneum or other surfaces, can penetrate those surfaces and can multiply *in vivo.* In addition to their thermotolerance and the ability to adapt to the physiochemical conditions of the host, these pathogenic fungi can also avoid the host defence mechanisms and so damage the host.

1.5.1 Invasion Processes

All dermatophyte species have the capacity to invade the stratum corneum of the epidermis and the follicular ostium of hairs while most other keratinophilic fungi have only limited ability to do so.

But in all, following the uptake, adherence and subsequent germination of an infectious propagule to a host substrate, colonisation of the affected substrate proceeds via both mechanical and enzymatic means. Several species exhibit differences in growth patterns thought to be related to differing nutritional factors and colonisation *in vivo* and *in vitro* can involve contrasing mechanical and enzymatic processes. The net result, especially in hair, can be very different.

1.5.1.1 Hair

The physical stages by which hairs are attacked *in vitro* by keratinophilic fungi are well documented (Barlow & Chattaway, 1955, Vanbreuseghem, 1950, English, 1963) with the principal mechanical mechanisms summarised as 1) cuticle lifting, 2) cortical erosion, 3) penetrating organs and colonisation of the medulla.

Following spore germination on or near a hair, germ tubes, following the edges of scales of the cuticle, encircle the hair, developing into a wide main hypha oriented longitudally along the hair. From this, lateral branches are sent out encircling the hair and arising from these lateral branches come flattened fronds of 'eroding mycelium' which penetrate between the cuticular cells and the outer layers of the cortex.

Immediately following lifting of the cuticle, cortical ersion occurs, carried out by the fronds of eroding mycelia with new fronds formed beneath the level of the original ones. Perforating organs, where formed, are basically a modified eroding mycelium consisting essentially of a column of short, wide cells, the lowest one cone-shaped and penetrating tangentially or radially into the hair, often projecting backwards from it.

In contrast, *in vivo* colonisation of the hair may occur in one of a number of invasion patterns. These patterns are commonly known as "Endothrix", where invasion by hyphae occurs within the hair shaft itself or "Ectothrix", where invasion also occurs within the hair follicle.

There are no eroding hyphae or perforating organs formed *in vivo* but the pattern of hair invasion along with the size of arthrospores formed by disarticulation of hyphae can often suggest the identity of the infecting agent.

1.5.1.2 Skin

The arthroconidia of dermatophytes appear able to adhere to all body surfaces including the face, back of hand, palm, leg and sole. Infection is established following contact, adherence and germination of a propagule. The germination of a suitable propagule may be seen by the formation of germ tubes and penetration longitudinally between layers and transversely between the thickness of the stratum corneum followed by the formation of hyphae.

In vivo the microscopic morphology of the invader is commonly of long, septate and branched hyphae which eventually disarticulate into arthrospores, completing the cycle. It is the horizontal extension which results in the clinically observable signs of peripheral expansion of the lesion. In contrast, *in vitro* colonisation leads to the development of the typical saprophytic morphology of the fungus (Baxter, pers. comm).

It should be recognised that the skin consists of two distinct parts, the epidermis or outer layer, which is the area to which invading dermatophyte species are generally physically confined and the dermis or inner layer. These are joined at the dermal-epidermal junction, via which *in vivo* the cells of the immune system must cross if they are to be effective. It has been observed that following the initial invasion of the skin, the fungus remains confined to the stratum corneum, while pathological changes occur in deeper layers of the epidermis and the dermis (Pilsbury *et a.,l* 1956), suggesting that pathological reactions are mediated by diffusible product(s) of the fungus. Cruickshank and Trotter, (1956) found that culture filtrates from *T. mentagrophytes* and *T. rubrum* affected bonds in the epidermal-dermal junction resulting in separation of the epidermis from the dermis. Thus the net result *in vivo* is the development of scales and vesicles.

1.5.1.3 Nails

The nails of humans are made up of a strong plate of hard keratin that grows out from a curved transverse groove. The floor of this groove is the germinal region of the nail plate and is therefore known as the nail matrix.

As the progeny of proliferating cells approach the dorsal surface of the nail, they are displaced distally and gradually transform into hard keratin, causing the nail plate to lengthen and strengthen (Cormack, 1993).

The horny layer of the distal nail plate and hyponychial groove may assist trapping of dermatophyte infective particles (Tsuboi *et al.*, 1994). The most common type of fungal nail infection is distal and lateral subungual tinea unguium, usually originating from fungal infection of the plantar and palmar surfaces of the feet and/or hands. Invasion of the hyponychial epidermis and subsequent invasion of the nail bed epidermis occurs. This in turn is followed by invasion of the most distal and lateral aspects of the nail plate (Ogawa *et al.*, 1998).

Infection of the nail bed epidermis results in thickening of the stratum corneum beneath the nail plate which causes a slight elevation of the nail plate from the nail bed. It is this change which clinically gives the involved nail an altered cream colour rather than the normal transparent appearance. Initally the dense keratin of the nail plate is not involved but becomes infected later (Ogawa *et al.*, 1998).

Dermatophytes tend to be orientated at random in hyponychial keratin, but lie parallel to the surface in the nail plate. Long septate and branched hyphae, with arthrospores, are the main microscopic features found in dermatophytic nail infections *in vivo*. Over time, dermatophytes create tunnels containing air within the nail plate and where the network of lacunae is sufficiently dense , the nail is opaque (Simpanya, 1994).

The dermatophytes commonly associated with tinea unguium are *T. rubrum* and *T. mentagrophytes*. The latter is normally seen only in toenail infections (Ogawa *et al.*, 1998). The main non-dermatophyte moulds involved in onychomycosis appear to be *S. brevicaulis & Scytalidium*, (Ellis, 1996) . However, there is considerable controversy on the significance of these moulds when identified in the presence of a dermatophyte (Ellis, 1996).

In vitro, nails are colonised by the 'saprophytic' phase of the fungus, although under certain cultural conditions, arthrospores can be induced (Baxter, pers. comm.).

1.5.2 Enymatic Invasion

1.5.2.1 Proteolytic Enzyme Production

The evidence for proteolytic enzyme involvement in the processes involved in colonisation of keratin has been established for some time (Davidson *et al.*, 1934). Observations of empty areas around perforating organs e.g. the mouth of a pit that is 2 or 3 times as wide as the actual organ within it, or a trough extending beyond the eroding mycelium it contains (English, 1963) are other examples.

Baxter & Mann (1961) examined the pattern of invasion of cut human hair *in vitro* by three dermatophytes (*T. mentagrophytes*, *T. rubrum and T. ajelloi*) and found variations in the keratinolytic ability of these species. *T. mentagrophytes* was the most keratinolytic and was seen to breakdown the hair keratin as evidenced by "gaps" around fungal structures. It has also been shown that the fungus was capable of causing extensive breakdown of keratin in the saprophytic stages and large numbers of granules and mitochondria were observed in the hyphae which were not observed in the other species. The authors suggested that this could be due to the higher enzymatic activity of this species. Mercer & Verma, (1963) looked at the invasion of sterile cut human hair *in vitro* by *T. mentagrophytes*. They also found that the process of hair invasion involved an enzymatic breakdown of keratin bundles with complete loss of the keratin.

Studies by Rashid *et al.*, (1996) confirmed the work of earlier researchers. *T. mentagrophytes* was seen to digest the endocuticle, causing detachment of cells from the underlying cortex.

Kunert and Krajci, (1981) studied the process of hair invasion by *M. gypseum in vitro* and found that the process of keratin degradation also had features of enzymatic breakdown, with some evidence of a mechanical effect of the hyphae on the cuticular cells.

The pattern of invasion of human hair by non-dermatophytic fungi shows many similar features. *Chrysosporium tropicum* is a soil-inhabiting fungus which when inoculated onto autoclaved hair formed perforating hyphae and showed all the characteristics suggestive of enzyme digestion (Yu *et al.*, 1972, A. Rashid *et al.*, 1996).

The breakdown of various compounds in keratin known to be recalcitrant to chemical proteolysis have been observed frequently and have confirmed that the dermatophytes posses the ability to degrade wool or hair and release sulphydryl compounds into the medium. When *T. rubrum & T. schoenleinii,* in separate experiments, were separated in the medium from ^{35}S radio-labelled wool by a permiable membrane Weary & Canby, (1969) showed that diffusable products produced by the fungi degraded the radio labelled wool

Further to this, other researchers were able to demonstrate keratinolytic abilities in a number of fungi e.g. Deshmukh *et al.*, (1982) were able to demonstrate via specrtrophotomeric methods, keratinolytic abilities in *Auxarthron conjugatum, Chrysosporium indicum, C. pannicola, T. ajelloi and M. gypseum in vitro* using human hair as the sole carbon and nitrogen source and later Apodaca *et al.*, (1989,1990) demonstrated, also via spectrophotomeric methods and SDS-PAGE, the keratinolytic abilities of *T. rubrum* in a keratin salts medium.

Evidence that a true secretion is involved has been established via measurements of alkaline phosphatase in culture filtrates (O'Sullivan & Matheson, 1971) as this enzyme can be used as an indicator of cell lysis. Studies have shown the appearance of the enzyme lags some 30 hours behind the appearance of keratinolytic proteases. When it appeared the levels were much less than those measured in cultures which had been allowed to proceed to the autolytic phase (O'Sullivan, 1970).

Following the confirmation of proteolytic enzyme production by dermatophytes, many researchers have reported purifying enzymes from a range of dermatophytes with many of the enzymes isolated found able to hydrolyse various proteins such as casein, elastin, collagen and keratin e.g Yu *et al.*, (1969) using spectrophotometric methods were able to measure the keratinolytic activity of proeteinases of *T. mentagrophytes* which was found able to hydrolyse guinea pig hair.

Further to this, other researchers have begun characterisation of enzymes produced in investigations of these proteinases as virulence factors during the pathogenesis of infection. (Simpanya, 1994, Palmer, 1995).

A number of these may be serine proteinses, which act by forming covalent complexes between the enzyme and substrate with the hydroxyl (OH) group from the serine amino acid making a nucleophilic attack on an acyl of the substrate leading to the formation of an ester bond between enzyme and substrate. Via hydrolysis this ester bond is broken along with the peptide bond present in the protein. In one recent example Mignon *et al.*, (1998) isolated via affinity chromatography and SDS-PAGE techniques a 31.5 kda protease from a clinical isolate *M. canis*. This protease was found to belong to the subtilisin-like serine protease family.

Some are cysteine proteinases which also form covalent bonds between enzyme and substrate but the attacking nucleophile is a sulphur atom from the cysteine side chain, with the reactions occurring being the same as those occurring for serine proteases.

Aspartic proteinases and metalloproteinases do not form covalent intermediates, with the catalytic apparatus of the aspartic proteinases thought to be two aspartic acid side chain residues with the carboxyl groups thought to cause cleavage of the peptide bond at low pH e.g.3-5. Metalloproteases however, contain a metal ion such as zinc which provides a strong electrophilic pull which participates in the attraction of a water molecule causing cleavage of the peptide bond (Stryer, 1995).

Evidence suggesting that the primary attack of dermatophytes on keratin rich material is not on keratin itself but on more readily degradable substances has been reported (Raubitschek, 1961, Ruffin *et al.*, 1976). Stahl and co-workers (1950) showed that the first wool proteins degraded by *M.gypseum* are the non-sulphur containing molecules. This was later confirmed by Hose (1976) who showed that release of ^{35}S from guinea pig hair previously radio-labeled *in vivo* occurred after the maximum release of peptide material assayed by chemical methods (Odds, 1991).

Substrate reduction as a key reaction in keratinolysis with thiosulphuric ester synthesis, has been deduced from histochemical tests made in hair perforations (Kunert, 1972) and has been shown to be a key reaction in the degradation of keratin by dermatophyte species. Synthesis of thiosulphuric esters from the disulphide bonds of the keratin can be explained by sulphitolysis reactions (Swan, 1957).

$$\underset{\text{disulphide}}{R\text{-}S\text{-}S\text{-}R'} + \underset{\text{sulphite}}{HSO_3^-} \rightarrow \underset{\text{thiol}}{RSH} + \underset{\text{thiosulphuric ester}}{RSSO_3^-}$$

According to the above equation, cystine (Cy - S - S - Cy) would be converted to S - sulphocysteine (Ruffin *et al.*, 1976, Kunert, 1972).

As long as sulphite is produced from protein-incorporated cystine, the disulphide bonds of keratin can be split according to the above equation and keratin denatured in this way.

However, regardless of the mechanism used, it is clear that the lytic capability of dermatophytes to produce proteinases for keratin substrates goes beyond that of other fungi. Chesters and Mathison (1963) showed that *T. ajelloi* could release amino acids from animal wool whereas trypsin had almost no activity in that assay. Other studies have provided strong indications of a molecular basis for host specifities and affinities between dermatophyte species and keratinaceous substrates (Odds, 1991).

Also under investigation is the number of proteases produced by a strain or strains of a species at any point in time, with some schools of thought indicating that a set number of enzymes are produced for a given strain (Simpanya, 1994, Palmer, 1995) while other researchers have found that the number of enzymes produced per strain will vary depending upon the culture conditions (Tucker *et al.*, 1991, Sparkes *et al.*, 1994, Papini *et al.*, 1996)

Some of the above findings strongly suggest that fungi are able to quickly adapt to given environmental conditions although little is known about the regulation of protease expression. Clues to regulation have been sought from the more extensive examination of proteinase regulation in other filamentous fungi and this led to the proposal of a model for the regulation of *T. rubrum* proteolytic activity by Apodaca and McKerrow (1989). This model suggests that whenever the fungus lacks carbon, nitrogen or sulphur, a group of general proteinases is expressed which initially act upon the non-keratinous proteins within the substrate, providing the above elements, which in turn has a feed back effect repressing general proteinases with keratinases induced by the presence of insoluble proteins such as keratin.

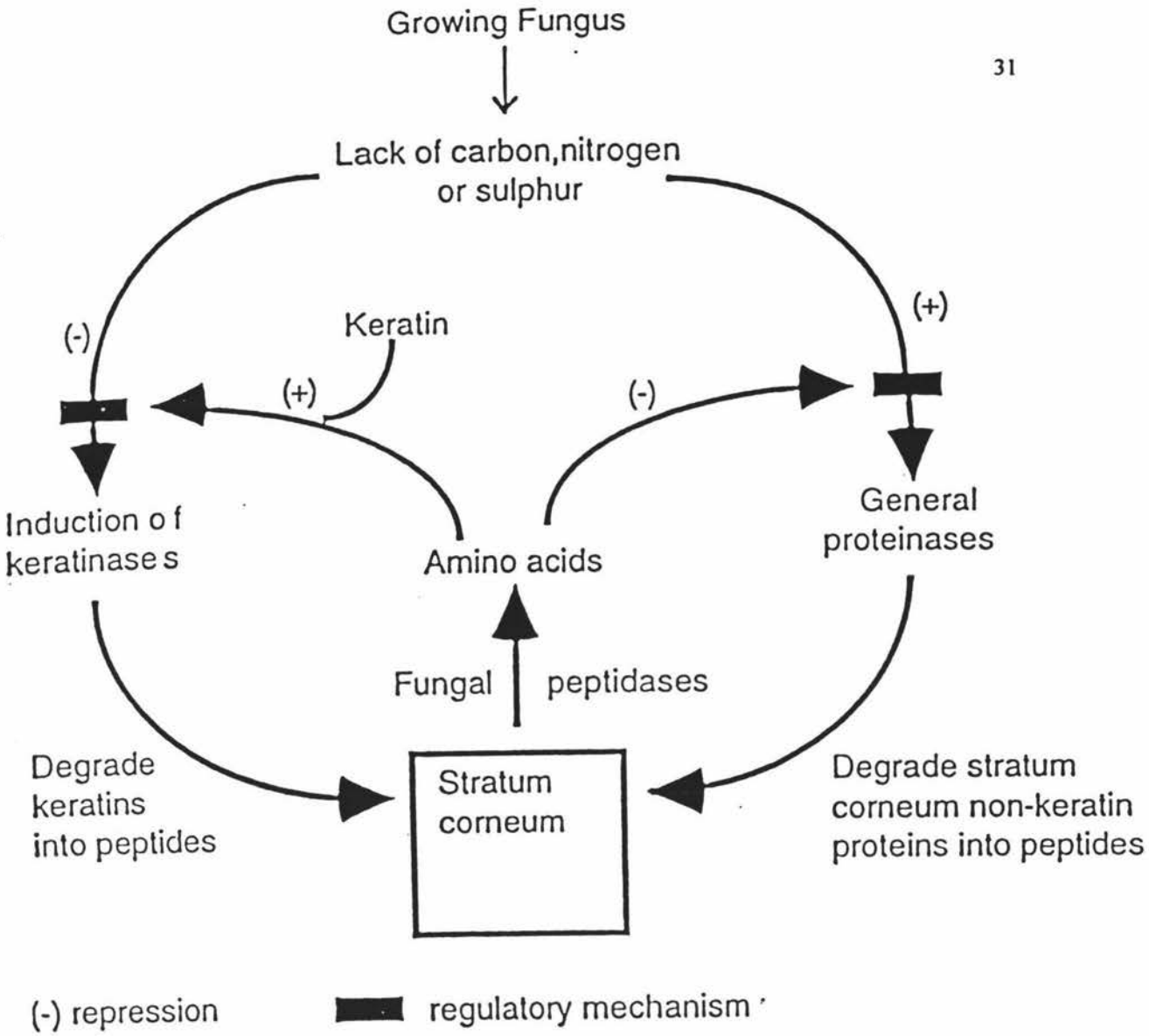


Fig 1.2 Proposed model for regulation of proteolytic activity by *T. rubrum.*

Apodaca & McKerrow (1989) also proposed that there were differences between the proteolytic enzyme expression between log and stationary phase cultures with those initially produced acting upon the non-keratinous proteins in the substrate. Once the stationary phase of growth occurs, proteinases are expressed constitutively, coinciding with disease which may directly or indirectly incite a host response such as inflammation & dermatophytosis.

While substrate specificity and regulation of proteolytic enzyme production is not well understood, the model proposed by Apodaca & McKerrow allows that the fungus is able to respond to its immediate environment, altering the initial production and secretion of proteases such that the immediate nutritional needs of the fungus are met from the substrates most readily available, as opposed to the production of one rigidly defined group of proteases.

1.5.2.2 SDS-PAGE Technique for Enzyme Analysis

Of the various techniques which can be used in enzyme analysis work, polyacrylamide gel electrophoresis (PAGE) has been used by a number of investigators (Takiuchi *et al.*, 1983,1984, Sanyal *et al.*, 1985, Apodaca *et al.*, 1989a, 1989b, 1990, Brahim-Granet *et al.*, 1996.). This technique gives good resolution of separate proteins obtained from samples of only 5 - 25 μg. Electrophoretic methods are able to simultaneously exploit differences in the molecular size of the protein involved and, if appropriate to the work being done, the charge on the enzyme/s of interest. Therefore PAGE separates out proteins on the basis of either size, charge or both.

In the past, high resolution has been achieved via disc electrophoresis utilising a synthetic gel medium of cross-linked polyacrylamide and systems of discontinuous buffers (Ornstein *et al.*, 1964, Arcus, 1970). Nowadays, the electrophoretic method most commonly used is thin-slab gel electrophoresis, on which several samples can be run at the same time allowing for a direct comparison of mobility (Scopes, 1987, Osterman, 1984). PAGE is advantageous as it can be carried out at a wide range of temperatures, and environmental conditions can be specified such that minimal damage occurs to the enzyme/s being examined.

The studies which have been reported utilise denaturing gels, the enzyme extract being treated with sodium dodecyl sulphate (SDS) prior to electrophoresis, so conferring an overall negative charge on the enzyme/s, with subsequent separations using this method thus carried out on the basis of molecular weight. This high-resolution method has two major advantages compared to native electrophoresis. One is that aggregates and insoluble particles which often cause bad results with native gels are solubilised by the SDS, the other is that mobility is related to polypeptide size, so an immediate indication of the molecular weight for each component is provided (Scopes, 1996).

Of particular value is the ability to utilise substrate co-polymerised polyacrylamide gels. These are a modification of Laemmli's (1970) procedure whereby suitable substrate is co-polymerised with the gel in order to assist visualising of enzyme activity. Again enzyme activity is localised by molecular weight.

These gels differ from the standard SDS-Laemmli gels in two ways (1) as mentioned earlier, the gels are made incorporating a suitable substrate of interest e.g. gelatin, within the polyacrylamide matrix and (2) the sample is mixed with a higher concentration of SDS to which is added sucrose and bromophenol blue as a tracking dye.

Despite the fact that the potential of separation of proteins of identical size is not possible with this system, it does give the sharpest overall resolution and cleanest zones of any method and by making a comparison with a mixture of standard polypeptides of known molecular weight, the whole gel can be calibrated in terms of mobility against size (Scopes ,1996).

There are no reducing agents such as β-mercaptoethanol or dithiothreitol added to the samples being analysed, nor are samples boiled, leading to irreversible denaturation of the enzymes of interest. However, standard polypeptides of known molecular weight are prepared using β-mercaptoethanol and boiled for approximately five minutes, then run on the same gel as the protein sample. This allows for the simultaneous detection of the enzymes of interest, along with determination of their molecular weight.

Following electrophoresis, the SDS is removed and the gel washed in the non-ionic detergent triton X-100 for 2 hours to allow for renaturation of the enzymes. (Heussen *et al.*, 1980, Asahi *et al.*, 1985) The gels are then incubated in a suitable buffer allowing for the breakdown of the gelatin by enzyme activity for 12 to 24 hrs., after which the protein band must be visualised.

Visualisation is commonly done by staining with dye of either the protein/s of interest (positive staining) or staining of the gel background with no staining of the protein/s of interest (negative staining) e.g. coommassie brilliant blue R-250 can be used, utilising a negative staining technique in which the main body of the gel retains the stain while areas where proteolytic activity occur in the gel matrix do not, leaving cleared areas visible as bands on the gel. Following the construction of a standard curve using the known standards, the molecular weight of each band can be calculated.

AIMS

1. To compare mycelial morphology of keratinophilic fungi in relation to spore production in stationary and shake culture and assess the possible establishment of a "pseudo-parasitic" condition.

2. To extend earlier studies of Simpanya (1994) and Palmer (1995) concerning proteolytic enzyme production by *Microsporum spp.*. to a range of keratinophilic fungi, dermatophytic and non-dermatophytic, to investigate and possible similarities in relation to keratin breakdown.

3. To assess the reproducibility of the substrate co-polymerised SDS-PAGE technique in the investigation of proteinases; to refine the technique as necessary and enquire into the consistency of results.

4. To investigate the influence of medium and culture conditions on proteinase production, particularly using wool containing media.

MATERIALS AND METHODS

2.1 Basic Mycological Technique

2.1.1 Basic Media

(a) Sabouraud's Dextrose Agar (SDA) - Oxoid premixed powder

Approximate composition

Glucose	40 g
Neopeptone	10 g
Agar	15 g
Sterile Distilled Water(SDW)	1000 ml

Mixed together while heating to boiling point, then autoclaved for 15 min at 120°C. Plates and/or slopes (Universal bottles) poured as required.

(b) Sabouraud's Dextrose Agar with Antibiotics (SDAXX)

To make 1L of SDAXX, the ingredients were mixed as above and the following added prior to autoclaving -

Chloramphenicol (Sigma)	0.05 g/10 ml ethanol(95%)
Cycloheximide (Sigma)	0.5 g/10 ml acetone

Chloramphenicol serves to suppress bacteria, while cycloheximide is known to inhibit many non-keratinolytic species of fungi. Combined they are commonly used in isolation media and are particularly useful when isolating dermatophytes and other keratinolytic species.

(c) Dilute Sabouraud's Dextrose Agar (Dil SDA)

Neopeptone (Difco)	1.0 g
Glucose (Serva D-Glucose)	2.0 g
Agar	15 g
SDW	1000 ml

Prepared and autoclaved as for SDA.

(d) Sabouraud's Dextrose Broth (SDB)

Neopeptone	10 g
Glucose	40 g
SDW	1000 ml

Prepared and autoclaved as for SDA.

(e) Lactrimel Agar (LMA)

Plain Flour	14 g
Skim Milk Powder	14 g
Honey	7 g
Agar	30 g
SDW	1000 ml

Mixed together while heating, autoclaved 12 min at 120°C.

Lactrimel agar is useful for rapid production of pigments and spores and will usually produce normal macroconidia in abundance in cultures in which macroconidia are not produced, or are scarce or atypical (Baxter & Rush-Munro, 1980). Antibiotics may also be incorporated if required.

(f) Lactrimel Broth (LMB)

As above but without agar.

When autoclaving Lactrimel media, a sedimentation of flour and skim milk powder may form. Careful shaking of the bottle upon completion of autoclaving will help resuspend the sedimented material. Some sedimentation of material is not prejudicial to the use of this medium.

(g) Basal Salts Medium (BS)

K_2HPO_4	1.5 g
$MgSO_4.7H_2O$	0.025 g
$CaCl_2$	0.025 g
$FeSO_4$	0.015 g
$ZnSO_4$	0.005 g
SDW	1000ml (1L)

Heated and mixed, then 50 ml aliquots transferred to 250 ml Erlenmeyer flasks and the flasks stoppered with non-absorbent cotton wool covered with tin foil. Autoclaved 20 min at 120°C (Deshmukh & Agrawal, 1985).

Any autoclaved media not immediately used was stored at 4°C until required.

2.1.2 Sterile Wool for Assay Cultures

Clean sheep's wool was rinsed and then soaked in soap and water for several days. The wool was rinsed with distilled water, squeezed to remove excess water and allowed to dry overnight at 37°C. Once dried, the wool was then soaked in ether for 48 hours to remove fatty material, the ether decanted off and the wool allowed to dry inside a fume cupboard overnight. Samples of 0.5g were packed into small paper bags and sterilised with ethylene oxide (88% CO_2, 12% ethylene oxide, 2 hrs at 35 kPa) by L.J. Eccles Ltd, Auckland.

2.1.3 Preparation of BS+Wool (BSW)

To each of the autoclaved Erlenmeyer flasks containing BS medium, a 0.5 g sample of ethylene oxide sterilised wool was aseptically added and the flasks re-stoppered.

2.1..4 Preparation of BS + Wool Autoclaved Cultures

To each of the Erlenmeyer flasks containing BS medium, a 0.5 g sample of ethylene oxide sterilised wool was aseptically added and the flasks re-stoppered following this the flasks containing the BS+Wool were autoclaved 20min @ 120°C.

2.1.5 Stain for Microscopy

Lactic acid	100 ml
Acid fuchsin	0.1 g

Acid fuchsin powder was dissolved in the lactic acid.

2.2 Sources of Isolates

2.2.1 Soil Sampling & Keratin Baiting

Soil samples were taken from various areas of Palmerston North and the surrounding districts. Duplicate samples of approx 50 g each, were placed into sterile Petri dishes and baited with sterilised keratin baits. The baits utilised were bird feathers, sheep wool and cat fur, these having been washed and autoclaved 15 min at 120°C. All soil samples were processed on the day of collection.

The baited samples were incubated at 25°C in the dark and moistened at intervals of 3-4 days to prevent the soil from drying. The plates were inspected daily for up to 6 weeks before being discarded.

The presence of keratinophilic fungi was confirmed by low power microscopic examination and the fungi isolated by inoculating fragments of colonised baits onto slopes of SDAXX. These slopes were incubated at 25°C, with further subculturing done as required.

2.2.2 Isolation from Animal Sources

Skin scrapings were taken from various animals using the edges of sterile microscope slides to prevent injury to the animals which would have occurred had a blade of any description been used.

The skin and hair was scraped directly onto SDAXX plates. The plates were then immediately returned to the laboratory and incubated at 25°C and keratinophilic species identified and isolated.

2.2.3 Other Sources

In addition to isolates obtained by the soil sampling and animal scraping techniques, other cultures were obtained from the Mycology Culture Collection (Mu cc) maintained at Massey University.

2.2.4 Identification

Keratinophilic fungi were identified according to standard methods on the basis of their macroscopic morphology on SDA and microscopic examination of the morphological characteristics of macro- and microconidia as appropriate, and the appearance of the mycelium.

Confirmation of identifications was made with reference to Rebell & Taplin, (1978) for *Microsporum & Trichophyton spp.*, Campbell & Stewart, (1987) for *Scopulariopsis sp.*, Carmicheal, (1962) and Cano & Guarro, (1990) for *Aphanoascus sp.*, Barron, (1968) for *Diheterospora sp.*.

From these identifications a selection was made for further study.

2.3 Preparation of Assay Cultures

2.3.1 Spore Suspensions

The selected species were aseptically inoculated onto slopes of solid media and incubated at 25°C in the dark. After 14 days, 5 ml of SDW was aseptically added to each slope and agitated by pipette to loosen spores on the culture surface. Then 4 ml of the water & spore mixture was removed and placed into sterile 10 ml bijou bottles, and kept at 4°C until required.

Spore suspensions in 100 μL aliquots were used to innoculate the SDB, LMB and BSW media utilised in studies of proteolytic enzyme production.

2.3.2 Spore Count using Improved Neubauer Haemocytometer Slide

An 'Improved Neubauer Chamber' was used to count spores in each suspension. This chamber has a ruled area of 3 mm^2 divided into 9 'large' squares, each 1 mm^2. The squares are further subdivided in different ways, e.g. the four corner large squares are each divided into 16 medium squares while the centre square is divided into 25 squares, each of which are further divided into 16 squares. The chamber depth is 0.1 mm.

Procedure:

1) Place a cover slip on the counting chamber
2) Add spore suspension carefully
3) Count spores in the 4 corner 'large' squares and the centre square. Take the mean number of spores per square and multiply by 10,000 to give the original concentration as spores/ml. As no dilutions were involved, this did not have to be taken into account.
4) The concentration in spores/ml was used to calculate the final concentration of spores inoculated into liquid cultures.

Each of the suspensions was counted twice and an average of the two counts was used to estimate spores/ml. Also, spores touching or crossing the top or right hand side of a square were counted while spores touching the bottom or left hand side of a square were not counted.

2.3.3 Measurement of pH of Culture Supernatants.

The pH of all culture supernatants were measured at regular intervals as an indicator of fungal growth. An aliquot of 4 ml of culture supernatant was aseptically pipetted into a 10 ml beaker and the pH determined using an appropriately standardised pH metre 28 or PHM210 standard pH metre (Radiometer Copenhagen, supplied by Watson Victor NZ Ltd.).

Readings of pH were taken for all liquid cultures, shake and stationary, as well as uninoculated media.

2.4 Assessment of Proteolytic Activity

2.4.1 Charcoal-Gelatin Disc Method

Materials

Charcoal-Gelatin discs (Oxoid)

Culture Supernatant(s)

Method

For an approximate indication of the proteolytic activity of samples, one charcoal-gelatin disc was incubated at 37°C with 2 ml of culture supernatant (SDB, LMB).

The potential activity was assessed by the release of the charcoal particles, either after 2 hours (high activity) or overnight (low activity) (Baxter, 1968).

This method, while being qualitative only, is reliable and very comparable to the gelatin hydrolysis plate method used by Baxter (1968) and Simpanya (1994).

2.4.2 Gelatin-Agar Plate Method

The method of Frazier (1926) was used to screen relative proteinase production by isolates.

Reagents

Solution A

Sodium chloride	5 g
Potassium dihydrogen phosphate	0.5 g
Dipotassium hydrogen phosphate	1.5 g
Sterile distilled water	100 ml

Ingredients were mixed and dissolved in the distilled water.

Solution B

Gelatin	4 g
Dextrose	0.05 g
0.8% Nutrient broth	5 ml
Sterile distilled water	400 ml

Ingredients were mixed together and then combined with Solution A. As the two solutions were being mixed and heated, 3% agar was added and pH adjusted to 7.0 using 1M HCL, if required, prior to autoclaving.

The medium was poured into petri dishes and allowed to set under UV light. Duplicate plates were inoculated with each isolate grown on SDA or LMA and incubated at 25°C for 7 days.

After 7 days incubation the plates were flooded with 5 ml of a solution of 15 g mercuric chloride dissolved in 20 ml HCL (conc) and 100 ml water.

The mercuric chloride was left for 15 min, to precipitate the undigested gelatin. A clear zone around the colony appeared surrounded by a cloudy precipitate of undigested gelatin. The remaining mercuric chloride was washed from the plate with water into a glass vessel for separate disposal, and all plates which had been in contact with the mecuric chloride were disposed of separately.

Measurement of the colony radius and the extent of gelatin hydrolysis away from the colony margin allowed expression of proteolytic activity as the ratio extent of hydrolysis :colony radius (Baxter, 1968, Simpanya, 1994).

2.5 Substrate Co-Polymerised SDS-PAGE Gels

The technique used for electrophoresis was the same as that used by Palmer (1995), which was a modification of that used by Simpanya (1994), based on the method of Laemmli (1970).

2.5.1 Reagents & Materials

(a) Resolving Acrylamide Solution

Acrylamide (BDH)	30.0 g
Bis-Acrylamide (BDH)	1.0 g
SDW	~70 ml**

Acylamide was added to approx 40 ml of SDW and stirred until dissolved. The bis-acrylamide was added, the preparation made up to a total volume of 100 ml with SDW and stored at 4°C.

(b) Stacking Acrylamide Solution

Acrylamide	30.0 g
Bis-Acrylamide	1.6 g
SDW	~70 ml**

Prepared as for Resolving Acrylamide Solution.

(c) Lower Tris-HCL Buffer (1.5 M & pH 8.8)

Tris (BDH)	18.10 g
SDS* (BDH)	0.04 g
SDW	~70 ml**

Tris was added to 60 ml of SDW and mixed until dissolved. The pH was adjusted with HCL, the mixture was brought up to volume and stored at 4°C.

(d) Upper Tris-HCL Buffer (0.5 M & pH 6.8)

Tris	6.05 g
SDS	0.04 g
SDW	~70 ml**

Prepared as for Lower Tris-HCL buffer.

*SDS:Sodium dodecyl sulphate

**Approximate volume required to bring total volume to 100ml.

(e) 5XTris-Glycine Reservoir Buffer (pH 8.3)

Tris	45 g
Glycine	216 g
SDS	15 g
SDW	2.75 L (total vol. approx 3 L)

Components were mixed together and stored at 4°C until required.

Dilute 300 ml 5X stock with 1,200 ml SDW for one electrophoresis run.

(f) Sample Buffer (SDS reducing buffer)

SDW	4.8 ml
0.5M Tris-HCl, pH6.8	1.2 ml
Glycerol	1.0 ml
10% (w/v) SDS	2.0 ml
0.05% (w/v) bromophenol blue	0.5 ml

Mixed together and stored at room temperature.

(g) TEMED: N.N.N'N'- Tetramethylethylenediamine (Sigma 99%)

(h) Incubation Buffer (pH 8.0)

Tris	1.50 g
$CaCl_2$	0.03 g
SDW	250 ml

The components were prepared as for Lower Tris buffer and stored at 4°C

(i) Ammonium Persulphate Solution

Ammonium Persulphate	0.1 g
SDW	1 ml

Once mixed, this solution was kept for no longer than 2 hours.

(j) Gelatin Solution

Gelatin (Type B, Sigma)	0.05 g
SDW	5.0 ml

Gelatin was dissolved in SDW by heating until a clear solution formed, then cooled.

(k) Triton X-100 Washing Solution (2.5%)

Triton X-100 (BDH)	25.0 ml
SDW	980.0 ml

Components were stirred until dissolved and stored at 4°C.

(l) Bromophenol Blue Solution (0.1%)

Bromophenol blue powder	0.05g
SDW	50 ml

The Bromophenol blue was added to the SDW, mixed and the solution stored at room temperature.

(m) SDS Solution (10%)

SDS powder (BDH)	10 g
SDW	90 ml

The components were mixed together and stored at room temperature.

(n) Stock Stain Solution (1%)

Coomassie blue R250	2.0 g
SDW	200 ml

Components were mixed well, then filtered through Whatman N°1 paper and stored at room temperature.

(o) Working Stain Solution

Stock stain solution	110 ml
SDW	390 ml
Methanol	500 ml
Acetic acid	100 ml

Components were mixed together and stored at room temperature.

(p) Destaining solution

SDW	520 ml
Methanol	450 ml
Acetic acid	30 ml

Components were mixed together and stored at room temperature.

2.5.2 Molecular Weight Markers

Broad range molecular weight markers were supplied by Bio-Rad and contained the following molecular weight proteins.

Myosin	200,000 Da
β-Galactosidase	116,250 Da
Phosphorylase a	94,400 Da
Serum Albumin	66,200 Da
Carbonic Anhydrase	45,000 Da
Trypsin inhibitor	31,000 Da
Lysozyme	21,500 Da
Aprotinin	6,500 Da

(a) Buffer for molecular weight markers (Standards buffer).

β-mercaptoethanol	25 μl
Stock Sample Buffer	475 μl

2.5.2.1 Construction of Standard Curve

As with other forms of plane chromatography, it is generally easier to measure solute retentions on the stationary phase as the **retardation factor R_f**, also called 'relative mobility' rather than retention volumes or times. The retardation factor is defined as the ratio of the distance moved by the sample component to the distance moved by the solvent front.

In this study, the size of each of the proteolytic bands was estimated by comparing their mobility in the polyacrylamide gels with that of the molecular weight standards run in the same gel.

$$\text{Retardation factor } (R_f) = \frac{\text{distance migrated by unknown protein or marker}}{\text{distance migrated by dye}}$$

The R_f values of the molecular weight markers were plotted against the $\log_{10}$ of the markers and a line of best fit drawn. From this the R_f's of the proteolytic bands were calculated.

2.5.3 Preparation of Gels

(a) Moulds

A Bio-Rad Protean II X1 cell assembly was used with outer glass plates of sizes 200 mm x 225 mm and inner glass plate sizes 200 mm x 200 mm, using 0.75 mm spacers. Petroleum jelly was used to help seal the joints.

(b) Resolving Gel

A resolving gel of 10% Acrylamide content was prepared as follows:

Lower Tris Buffer (pH 8.8)	10.00 ml
Resolving Acrylamide Solution	13.40 ml
SDW	12.60 ml
0.1% Gelatin	4.00 ml
Ammonium persulphate soln.	0.20 ml
TEMED	0.02 ml

Buffer, acrylamide, SDW and gelatin solution were mixed together in a 250 ml conical flask by swirling carefully, trying not to create air bubbles. The ammonium persulphate and TEMED were added last and the vessel swirled. The suspension was pipetted with a 5 ml pipette into the prepared mould. A small volume of SDW (~2 ml) was then carefully distributed over the surface of the running gel to create anaerobic condition required for complete polymerisation to occur.

Polymerisation took about 45 min, following which the SDW was decanted off and the stacking gel was poured onto the resolving gel.

(b) Stacking Gel

A 30% acrylamide/1.6% bis-acrylamide stacking gel was prepared as follows:

Upper Tris buffer (pH 6.8)	2.50 ml
Stacking acrylamide solution	1.30 ml
SDW	6.10 ml
Ammonium persulphate soln.	0.05 ml
TEMED	0.01 ml

The buffer, acrylamide and SDW were mixed together by swirling, again trying not to create air bubbles. The ammonium persulphate and TEMED were

added to the mixture and approx 1 ml used to wash the surface of the polymerised resolving gel after the SDW had been decanted off.

The mould was then filled with the stacking gel mixture to 5mm from the top of the mould. A 15 tooth comb was inserted to a depth of 25 mm and the gel left until polymerisation had taken place.

After the stacking gel had polymerised the comb was carefully removed and the wells washed out with SDW.

The moulds were then clipped onto the water jacket and placed in the buffer chamber. Tris Glycine buffer was added to the upper buffer tank and a check made for leaks before the lower buffer tank was filled.

N.B. The stacking gel does not contain gelatin substrate.

The Bio-Rad Protean II system allows for between one and four copolymerised gels to undergo electrophoresis at the same time. This ability is useful when dealing with large numbers of samples.

2.5.4 Sample Preparation

A 1 ml sample of culture filtrate was aseptically autopipetted into an Eppendorf tube and kept on ice while other samples from other species/strains were taken. The 1 ml samples were spun in a Micro-Centaur Centrifuge for 10 min at 4°C.

For each sample, following centrifugation, 75 μl of culture filtrate was taken from the top of the sample and added to 25 μl of sample buffer, mixed and 25 μl of mixture was added to the acrylamide stacking gel well.

2.5.4.1 Preparation of Molecular Weight Markers (Standards)

Standards Buffer	19 μl
Standards	1 μl

The components were mixed together in an Eppendorf tube, then the tube containing the mixture was boiled for 5 minutes and a sample of 10μl micropipetted into the acrylamide stacking gel well.

2.5.5 Gel Electrophoresis, Staining and De-staining

The samples were loaded into the acylamide wells at 4°C. Once loaded, the electrophoresis unit was connected to a power unit (Bio Rad model 500/200). The gels were electrophoresed at approx 20mA/gel, i.e 40mA for two gels. If three or more gels were being electrophoresed, then 18 mA/gel was used to prevent too fast a run.

At 4°C the bromophenol blue in the sample and standard buffers allowed the progress of electrophoresis to be monitored. Electrophoresis was performed until the dye reached approx 1.5 cm from the bottom (between 4.5 & 5.5 hrs).

At the end of the run the power source was switched off and the gel removed from the mould and soaked in 150 ml of 2.5% Triton X-100 for 1 hour with gentle shaking at room temperature to neutralise the SDS. The Triton X-100 was removed using a vacuum aspirator, the aspirator facilitating the change of solutions with minimal physical contact with the gel.

Initially, following removal of Triton X-100, the lane in which the standards were run was removed and soaked overnight in incubation buffer separately to the rest of the gel to prevent loss of individual standards (Palmer 1995). However, this was eventually found to be unsatisfactory, as processing distortions occurring in the gel were not occurring in the standard lane, giving rise to inaccurate calculation of molecular weights of proteolytic enzymes visualised.

In order to overcome the loss of markers during incubation and to maintain proteolytic activity , the gels were left for 4 hours in approx 200 ml incubation buffer at 37°C with gentle shaking. Following this, the shaker was switched off and the gels were left overnight in incubation buffer. This is a modification of the system initially used by Apodaca and McKerrow (1989a)

The gel was then stained at room temperature with Coomassie blue stain for 1 hr with gentle shaking.

Once stained, the gel was destained with destaining solution until visualisation of proteolytic bands was reached and the gel then immersed in water and documented as soon as possible.

RESULTS

3.1 Environmental Sources of Fungi

3.1.1 Keratinolytic Fungi from the Soil

Of the 15 soils sampled 10 (66.6%) yielded fungi able to be identified as keratinolytic.

The species *M. gypseum & T. ajelloi* were the most commonly found, forming 84.2% of the keratinolytic population isolated.

A strain initially identified as *Chrysosporium keratinophylum* was isolated from the Himitangi Beach sample. However, the late and consistent development of cleistothecia by this isolate led to its renaming as *Aphanoascus terreus.*

A strain of *Paeciliomyces* was also isolated from the Rangiotu Farm sample R1. This is commonly isolated from bird feathers and other fur materials, but this species was not used in further studies as its lytic abilities are uncertain.

Table 3.1 shows the origins of the fungal species isolated from soil samples.

Table 3.1 Keratinolytic Fungi Isolated from Soil by the Keratin Baiting Technique

CODE	SAMPLE ORIGIN	SOIL TYPE	DAYS TO VISIBLE COLONIES	IDENTIFIED KERATINOLYTIC SPECIES
A1	Front Science Tower D	Loam	7	*T. ajelloi*
A2	Front Science Tower D	Loam	5	*T. ajelloi*
B1	Ag Hort Building	Loam	15	*T. ajelloi*
B2	Ag Hort building	Loam	7	*T. ajelloi*
C1	Lakeside	Loam	15	-
C2	Lakeside	Loam	15	-
D1	Vet Tower Public Entry	Loam	5	*M. gypseum*
D2	Vet Tower Public Entry	Loam	5	*M. gypseum*
E1	Monro Hill Entry	Loam	10	*M. gypseum*
E2	Monro Hill Entry	Loam	10	*M. gypseum*
F1	Shearing Yards	Sand	9	*M. gypseum & T. ajelloi*
F2	Shearing Yards	Sand	9	*M. gypseum & T. ajelloi*
G1	Chesham Rd. Foxton	Sand/Peat	5	*M. gypseum & T. ajelloi*
G2	Chesham Rd. Foxton	Sand/Peat	5	*M. gypseum & T. ajelloi*
H1	Aokautere	Clay	8	*M. gypseum & T. ajelloi*
H2	Aotkautere	Clay	8	-

Table 3.1 cont.,

J1	Himitangi beach	Sand	5	*A. terreus*
J2	Himitangi beach	Sand	9	*A. terreus*
R1	Rangiotu farm	Sand/Peat	7	*Paecilomyces sp.*
R2	Rangiotu farm	Sand/Peat	7	*M. gypseum*
S1	Feilding saleyards	Clay/loam	7	*M. gypseum*
S2	Feilding saleyards	Clay/loam	7	*M. gypseum*
T1	Taupo Showgrounds	Clay/Pumice	-	-
T1	Taupo Showgrounds	Clay/Pumice	-	-
U1	Massey Computing	Loam	7	-
U2	Massey Computing	Loam	7	-
V1	Bunnythorpe	Clay	7	-
V2	Bunnythorpe	Clay	7	-
XG1	Moutua milkshed	Sand/loam	-	-
XG2	Moutua milkshed	Sand/loam	-	-

3.1.2 Keratinolytic Fungi of Animal Origin

Three animals with obvious lesions were sampled for fungi. These were a kitten and a very old dog, from an animal shelter and a young cattle beast, from a farm at a different locality.

The kitten was found to be infected with a strain of *M. canis.* (Strain "A")

The elderly dog was initially also thought to be infected with *M. canis.* However, the lesions on this animal's body more closely resembled open sores and eventually yielded *S. brevicaulis.* This animal was in a severely compromised condition when it arrived at the refuge and subsequently died. The fungus was isolated from scabs inoculated onto a blood agar plate.

The cattle beast was thought to infected with a *Trichophyton sp.* When the skin scraping was taken, however, the species isolated was *M. cookei.*

Also, five human volunteers had skin scrapings removed from lesions confirmed by medical diagnosis as dermatophyte infections, but as treatment for the conditions had commenced it was not possible to isolate dermatophytes from these sources.

Table 3.1.2.1 Keratinolytic Fungi Isolated from Animal Sources

SAMPLE ORIGIN	KERATINOLYTIC SPECIES ISOLATED
Kitten	*M. canis*
Cow	*M. cookei*
Dog	*S. brevicaulis*
***Human**	-

* Treatment of obvious fungal skin conditions had commenced.

3.1.3. Isolates Selected.

Fungi to be used in further studies were selected from the above soil and animal samples and the Massey University Culture Collection (Mucc).

Table 3.2 Isolates Utilised for Study, Sources and Codes

Species/strain	Source	Mu Code	Study Code
M. canis	Kitten	-	A
M. canis †	*Mu cc	822	1
M. canis †	Mu cc	834	2
M. canis †	Mu cc	855	3
M. cookei	Cow	-	K
M. cookei	Mu cc	192H	C
M. cookei †	Mu cc	773	4
M. cookei †	Mu cc	788	5
M. cookei †	Mu cc	841	6
M. gypseum	Soil	-	B
M. nanum	Mu cc	-	L
T. mentagrophytes var mentagrophytes	Mu cc	-	D
T. mentagrophytes var erinacei	Mu cc	-	M
T. ajelloi	Soil	-	E
T. terrestre	Mu	-	J
S. brevicaulis	Dog	-	F
A. terreus	Soil	-	H
C. keratinophylum	Mu cc	12985	I
Diheterospora chlamydosporium.	Mu cc	804	7
Diheterospora chlamydosporium.	Mu cc	809	8

*Mu cc = Massey University culture collection

† = Species introduced for comparisons and later used as +ve controls

3.2 Morphological Aspects of Selected Fungi in Stationary and Shake Culture Modes.

Studies by Raubitschek (1955) and Evron - Maoz *et al.* (1960) of fungi grown in shake cultures found this method made the fungus produce hyphae, arthrospores and chlamydospores resembling the parasitic life phase of the fungi found in scales, hair and nails. This growth type is frequently referred to as "pseudo-parasitic"(Evron - Maoz *et al.*, 1960).) In this series of experiments, shake culture was used to attempt the induction of the parasitic phenotype, with a view to investigating enzyme production in this growth phase.

Initially, the morphology of the mycelium developed by selected fungi in shake culture was compared to that in agar culture.

3.2.1 *Microsporum canis* (A)

Plate 3.2.1 a *M. canis* - Colony on SDA, 21days, 25° C.

Rapid growth produces a cottony fluffy mycelium with a bright yellow pigment often seen in the peripheral growth. The reverse is golden yellow.

Plate 3.2.1 b *M. canis* - Photomicroscopy (x400) above colony.

Macrospores large, thick walled and spindle-shaped, 15-20 μm x 42.5-60 μm with between 2 - 15 septa., the wall roughening with age. Microspores scarce, approx 2.5 μm x 4.5 μm, clavate and produced at both hyphal tip and on short pedicels on lateral surfaces of hyphae.

Plate 3.2.1 c *M. canis* - Colony grown in BSW, shake culture, 21 days, 25° C.

Growth was not initially visible, but became visible over the 21 day period, with mycelial growth apparent on the surface of the wool exposed to air.

Plate 3.2.1 d *M. canis* - Photomicroscopy (x400) above colony.

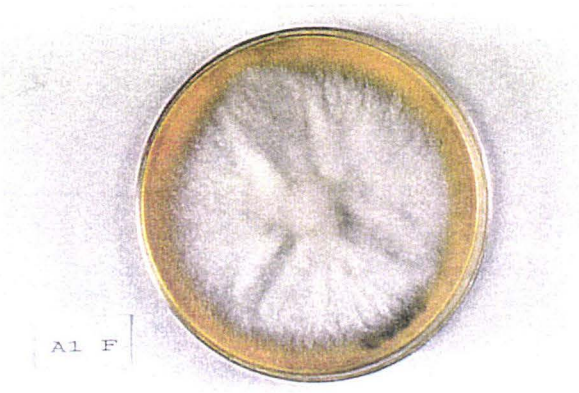
There was extensive mycelial growth but no macro or microspores were visible with this culture mode.

Plate 3.2.1 e *M. canis* SDB shake culture 21 days, 25° C.

Formation of circular pellets occurred within 7 days of innoculation of spores into culture vessel; by 21 days, numerous pellets had formed.

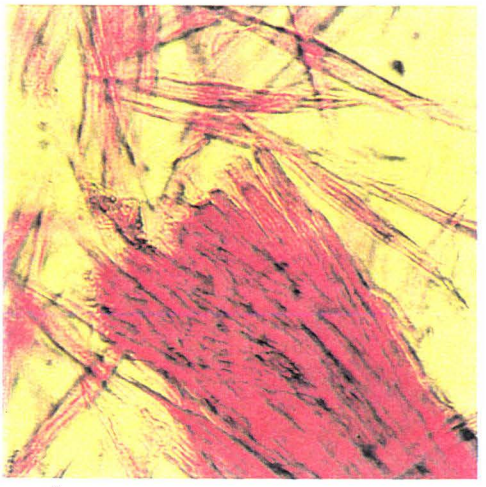
Plate 3.2.1 f *M canis* - Photomicroscopy (x400), above colony.

Extensive hyphal development, with what seems to be intercalary swelling at hyphal divisions. There was no apparent micro- or macrospore development for this culture mode.



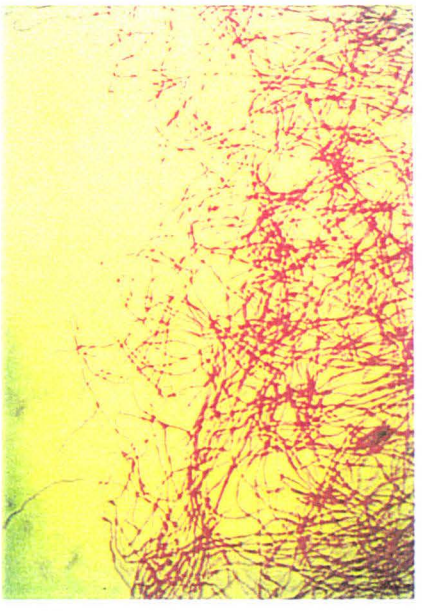
a

b



d

e



f

Plate 3.2.1g *M. canis* SDB, stationary culture, 21 days, 25° C.

No pellet formation was seen for this culture mode. Instead, a large mycelial mass formed over the surface of the nutrient with the submerged area being opaque while the surface growth was white in colour and of velvety appearance.

Plate 3.2.1h *M. canis* Photomicroscopy (x400) above colony

Extensive hyphal growth and expansion can be seen along with some macrospore development. Microspores did develop although not as abundantly as in other culture modes.

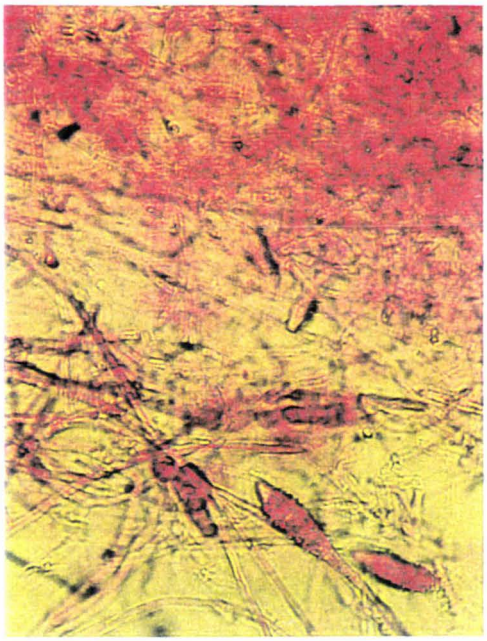
Plate 3.2.1i *M. canis* BSW, stationary culture, 21 days, 25° C.

The submerged areas of wool were enveloped in an opaque mycelial mat, with white, velvety textured growth seen on the surface.

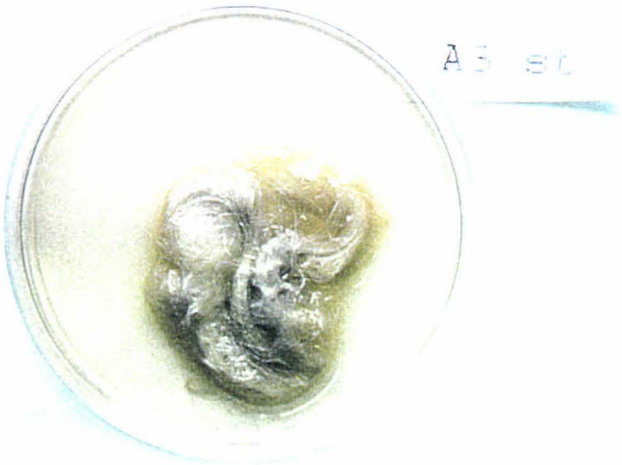
In general, the morphology of this strain did not vary much with variation in culture conditions. There was extensive mycelial development with all culture modes and while macrospore and microspore development was not visible in all cultures, where it was visible, it was typical for the species.

The non appearance of saprophytic spores in the shake culture along with the extensive hyphal development seen in the shake culture was typical for each of *M. canis*, *M. cookei* and *M. nanum* in this series of experiments, while *M. gypseum* exhibited other growth morphologies, which are examined in more detail under that species heading.

g



h



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3.2.2 *Microsporum cookei (K)*

Plate 3.2.2 a *M. cookei* - Colony grown on SDA, 21 days, 25° C.

Surface growth coarse and granular, tan to brown coloured with centre of colony often raised with white mycelia. Reverse, tan/ brown, becoming deep red/purple over time (~2 wks).

Plate 3.2.2 b *M. cookei* Photomicroscopy (x400) above colony.

Septate hyphae producing both macrospores and microspores abundantly. Macrospores - Oval shaped, more rounded than the macrospores of *M. canis,* thick walled, septate, 10 - 15 μm x 30 - 60 μm with between 2 & 10 septations.

Microspores - Produced abundantly, clavate, 2 - 8 μm x 2 μm, bourne along lateral edges and tips of hyphae.

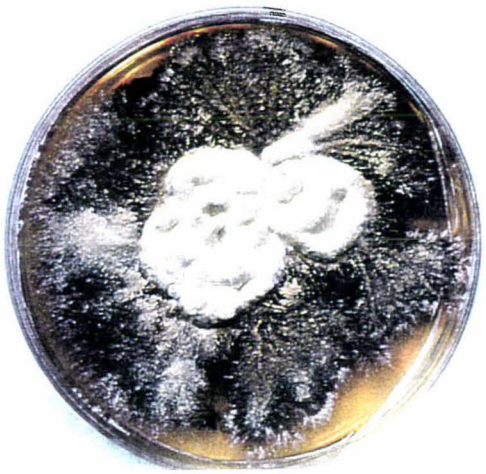
3.2.2 c *M. cookei* - SDB, shake culture, 21 days, 25° C.

Extensive pellet formation and pigment production was seen in this culture.

Fig 3.2.2 d *M. cookei* - BSW, stationary culture, 21 days, 25° C.
Mycelial formation apparent on surface of wool, along with pigment production.

Fig 3.2.2 e *M. cookei* Photomicroscopy (x400) above colonly

Macrospore formation has occurred, in submerged as well as surface areas of the colony.



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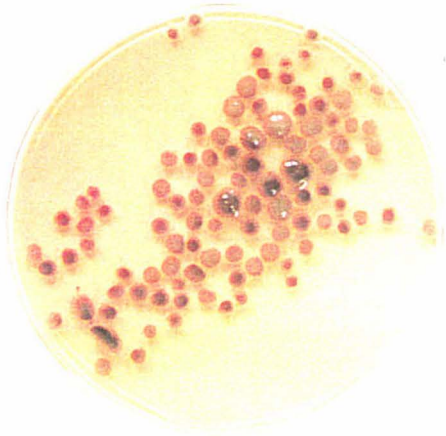

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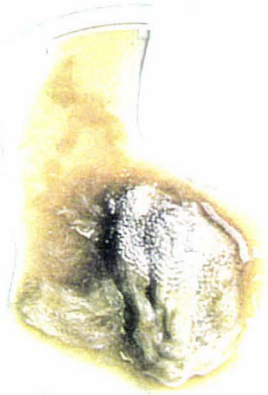


d

e

3.2.3 *Microsporum gypseum (B)*

Plate 3.2.3 a *M. gypseum* - SDA, 21 days, 25°C.

Rapid growth occurred on solid media. The colony was buff to dark lemon, with an uneven powdery surface. The reverse was bright yellow, although some colonies produced thin veins of deep red or brown colour.

There was a very strong odour, similar to that of rotting plant debris associated with this species throughout the course of these trials.

Plate 3.2.3 b *M. gypseum* Photomicroscopy (x400) above colony.

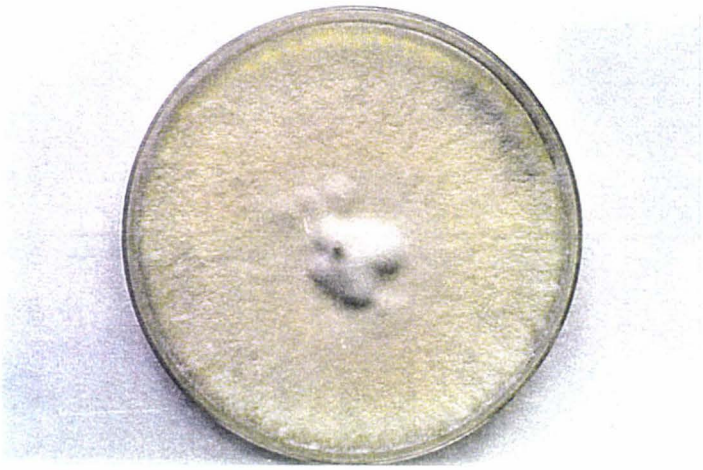
Thin walled, bullet shaped macrospores, 25 - 60 μm x 7.5 - 15 μm with 4 - 6 septations were abundantly produced, along with raquet hyphae. Smaller, clavate microspores of approx. 2.5 -3 x 4 - 6 μm were also produced.

Plate 3.2.3 c *M. gypseum* SDB, shake culture, 21 days, 25°C.

Large bright yellow pellets were formed in this culture mode and as with other strains, only the surface exposed to air developed a visible white mycelium.

Plate 3.2.3 d *M. gypseum* Photomicroscopy (x400) above colony.

Extensive segmented hyphal growth with no micro- or macrospores seen. The hyphal expansion was somewhat larger than that typically seen in arthospore development. The overall morphology, however, could be described as psuedo-parasitic in appearance.



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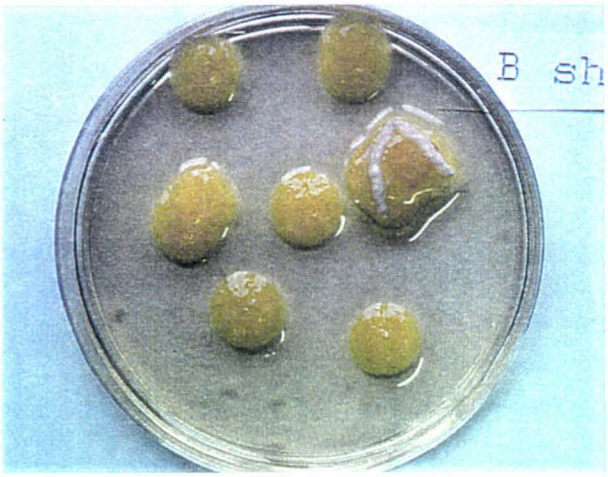

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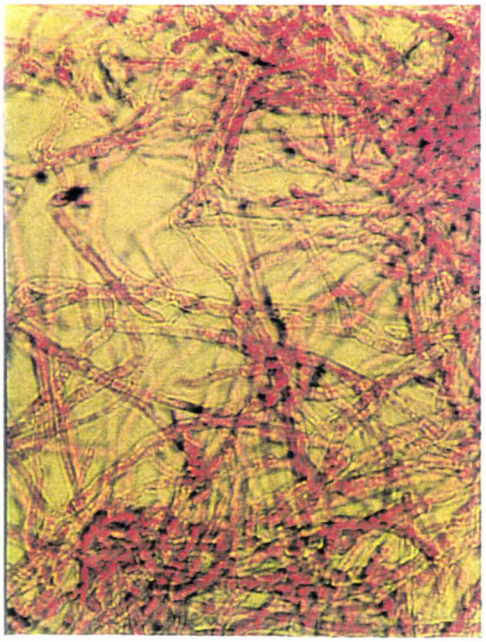


d

Plate 3.2.3 e *M. gypseum* SDB stationary culture 21 days 25°C

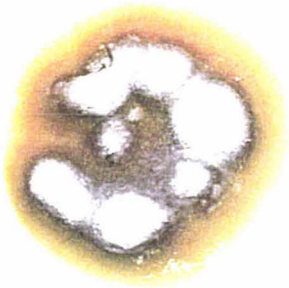
Surface growth was typical of that seen in solid agar cultures while the submerged growth was observed as a brownish pigmented gelatinous mass.

Plate 3.2.3 f *M. gypseum* Photomicroscopy (x 400) above colony.

This plate, as with plate 3.2.3d, shows this species exhibiting characteristics commonly attributed to the 'psuedohyphal' growth morphology with segmented hyphae and chlamydospore-like structures clearly visible.

Plate 3.2.3 g *M. gypseum* Photomicroscopy (x400) SDB, stationary culture 21 Days, 25°C.

This plate again shows the atypical microscopic morphology. Both microspore and macrospore development was abundant if somewhat atypical.



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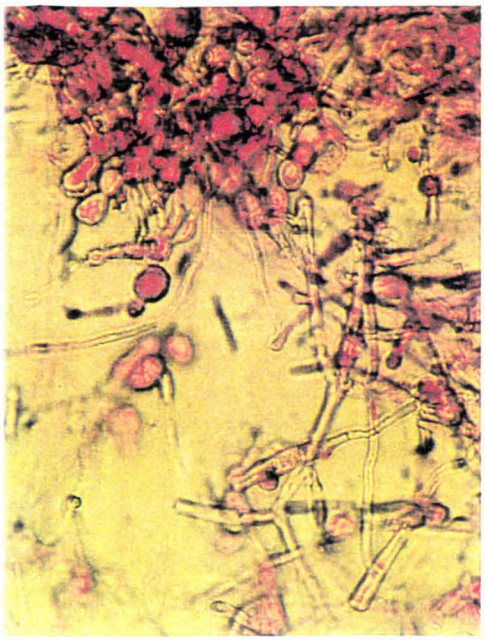

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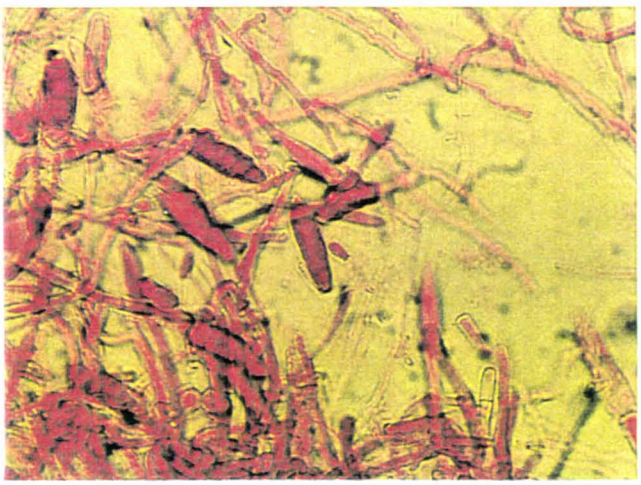


g

3.2.4 *Trichophyton ajelloi (E)*

Plate 3.2.4 a *T. ajelloi* - SDA, 21 days, 25°C.

Rapid growth was encountered on solid media, seen as an even powdery surface with a raised centre. Colony colour may be lemon to dark gold with light yellow to white margin.

The reverse is initially colourless, but a dark blue interspersed with dark red pigment develops over time. There is also frequently seen on the reverse, an 'age-ring' formation, similar to that seen in tree bark.

Plate 3.2.4 b *T. ajelloi* Photomicroscopy (x400) above colony.

Long oval to cigar shaped smooth , thick walled, macrospores, 20 - 65 μm x 5 - 10 μm containing 5 to 12 cells. These spores are slightly longer than those found in *Microsporum spp.* This fungus produces abundant macrospores but has only infrequently been documented as producing microspores, small, pear shaped microspores, but these were not found in the strains isolated during this study.

Plate 3.2.4 c *T. ajelloi* SDB, shake culture, 21 days, 25° C.

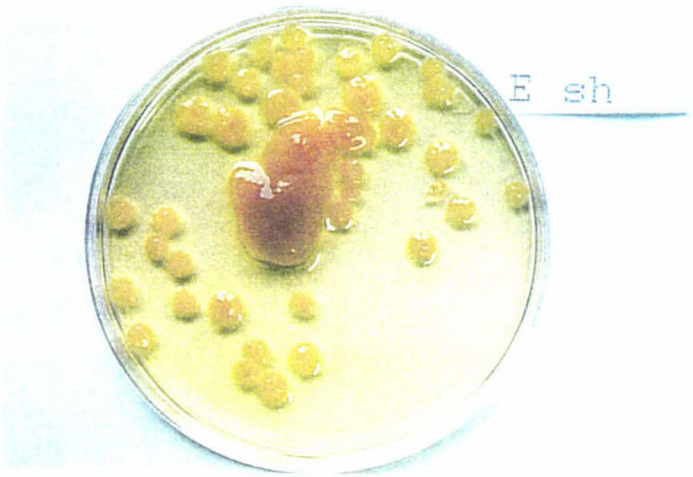
Numerous pellets formed, bright yellow.

Plate 3.2.4 d *T. ajelloi* BSW, stationary culture, 21 Days, 25° C.

Submerged wool held in gelatinous mass, with pigment diffusion throughout medium. While surface growth was visible in areas exposed to air.

a

b



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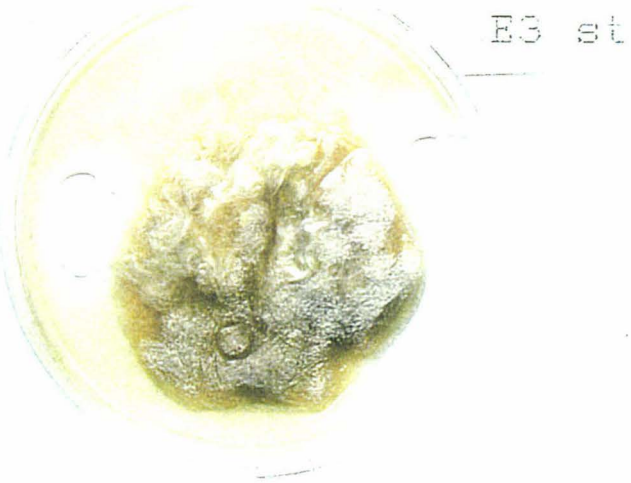


d

3.2.5 *Trichophyton mentagrophytes var mentagrophytes (D)*

Plate 3.2.5 a *T.mentagrophytes var mentagrophytes*, SDA, 21 days at 25°C

Rapid growing surface mycelia mostly flat with the centre somewhat raised, cream to yellow powdery to granular in appearance. Reverse a tan to golden yellow colour.

Plate 3.2.5 b *T.mentagrophytes var mentagrophytes* Photomicroscopy (x400) above colony.

Microspores small, 2-3 x 3-5 μm , clavate in shape, produced in large numbers along the lateral edges of hyphae, both singly but more commonly in clusters.

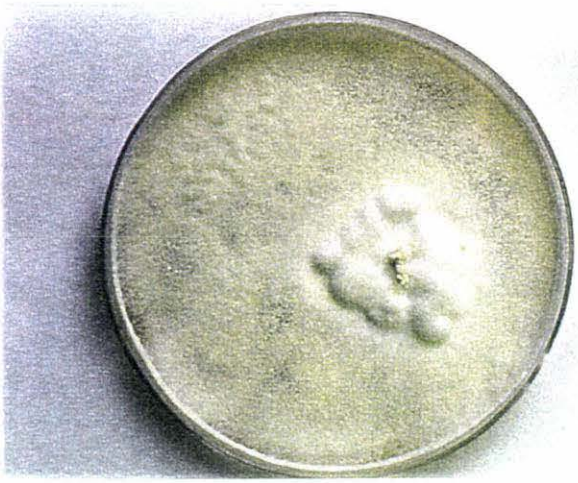
Macrospores were scarce in this strain, but when produced were between 20 -25 x 50-60 μm long, cigar shaped with thin walls and multicelled. Septate hyphae, branching and spiral forms frequently present.

Plate 3.2.5 c *T. mentagrophytes var mentagrophytes.* BSW, shake culture, 21 days.

Mycelial growth in this media was seen as an opaque gelatinous mass which encompassed the wool sample.

Plate 3.2.5 d *T. mentagrophytes var mentagrophytes.* Photomicroscopy (x400) SDB, stationary culture, 21 days.

Extensive hyphal development was seen with this culture mode, somewhat similiar to that seen in the *Microsporum spp..*, However, small nodules can be seen extending outwards form the lateral edges of the hyphae. This morphology was reproduced consistently with this culture mode.



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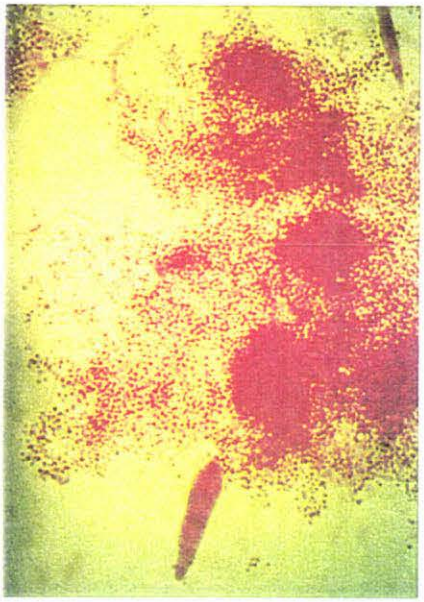


b

c

d

3.2.6 *Scopulariopsis brevicaulis (F)*

Plate 3.2.6 a *S. brevicaulis* - Colony grown on SDA, 21 days, 25° C.

Slow growing, this strain produced a thick, raised, coarsely granular brown colony, fading to white/cream at the edges, with some white tufts appearing in places. Extensive folding of the mycelial mat occurred. Reverse, brown.

Plate 3.2.6 b *S. brevicaulis* Photomicroscopy (x400) above colony

Long, extensively branced septate hyphae, up to 10 µm in diameter, with spores produced terminally from conidium - producing cells called annellides, which elongate slightly prior to the production of each new spore with the oldest spore found at the end of any chain formed.

Spores are lemon - shaped to round, 7.5 µm ave, with a thick outer wall enclosing the spore. The outer wall also appears to roughen with age.

Plate 3.2.6 c *S. brevicaulis* - BSW, shake culture, 21 days, 25° C.

Gelatinous mass of brown mycelial growth apparent. Pigment diffusion through medium.

Plate 3.2.6 d *S. brevicaulis* Photomicroscopy (x400) above colony

No apparent change in hyphal/spore morphology in this culture mode.

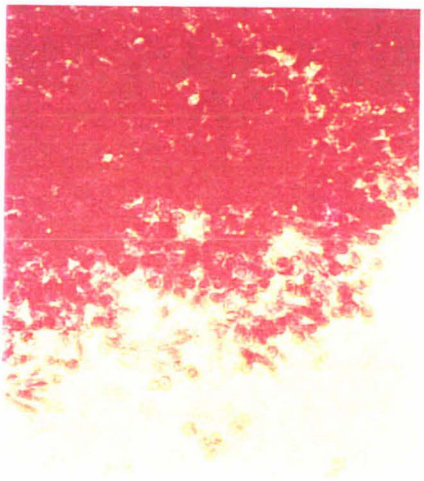
Plate 3.2.6 e *S. brevicaulis* - SDB, stationary culture, 21 days, 25° C.

Growth sparse with white, waxy, irregular pellets formed in this culture mode.

Plate 3.2.6 f *S. brevicaulis* - Photomicroscopy (x400) above colony.

Microscopic growth morphology was typical for this species, despite differences in gross colony morphology.

a



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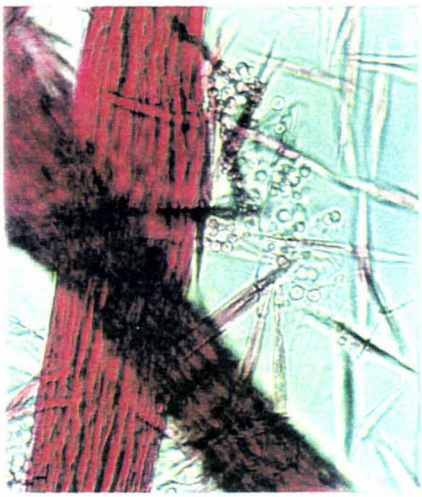

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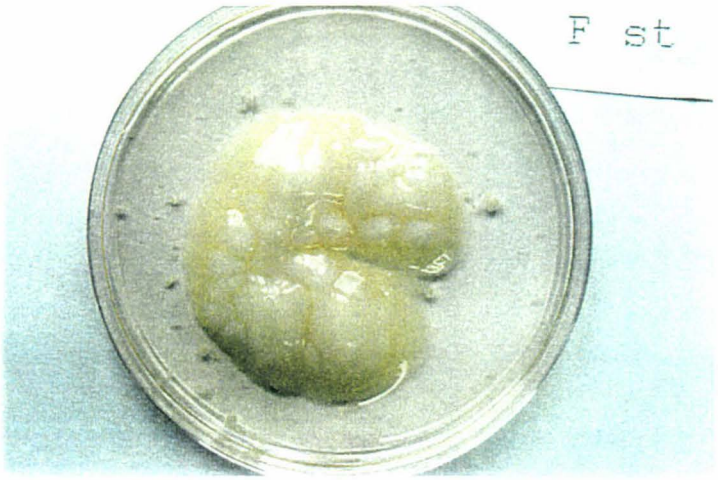

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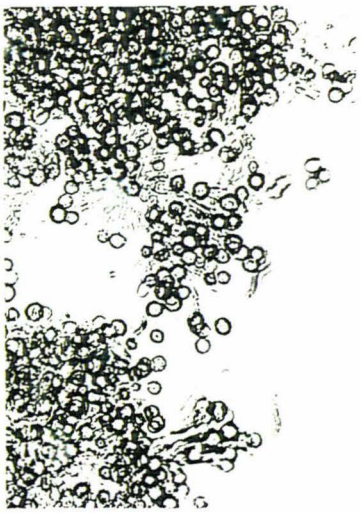


f

3.2.7 *Aphanoascus terreus (H)*

Plate 3.2.7 a *A. terreus* - Colony grown on SDA, 14 days at 25°C.

Surface growth velvety white to pale cream colour, radially extending folded appearance. Reverse is pale yellow to grey.

Plate 3.2.7 b *A. terreus* - Colony grown on SDA, 21 days at 25°C.

Again, surface growth was velvety white to pale cream colour, but cleistothecial development was seen in the form of raised black granules. Microscopic examination of the granules confirmed they were cleistothecia.

Plate 3.2.7 c *A. terreus* - Photomicroscophy (x400) 14 day colony.

Long septate hyphae, with microspores clavate, bourne both at hyphal tip and on short and long pedicels along the lateral edges of hyphae, between 1.5 μm x 1.5 -3.0 μm typical of *Chrysosporium sp.*

Plate 3.2.7 d ***A. terreus*** BSW, shake culture, 21 days.

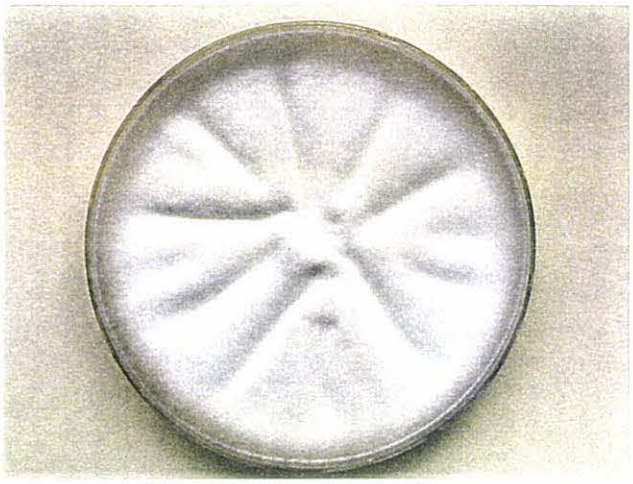
Mycelial mat formed as a gelatinous mat surrounding the wool, some slight pigment production.

Plate 3.2.7 e ***A. terreus*** BSW, Photomicroscopy (x400) above colony,

Microscopic examination of colony showed no differences to that seen in solid media culture.

Plate 3.2.7 f ***A. terreus*** BSW, stationary culture, 21 days.

The main mass of the submerged wool degraded quite rapidly. However, non-submerged wool remained intact, with cleistothecal development plainly visible on the surface.



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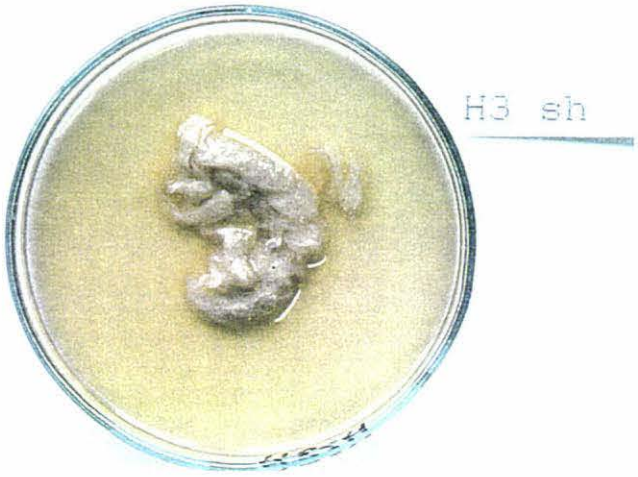

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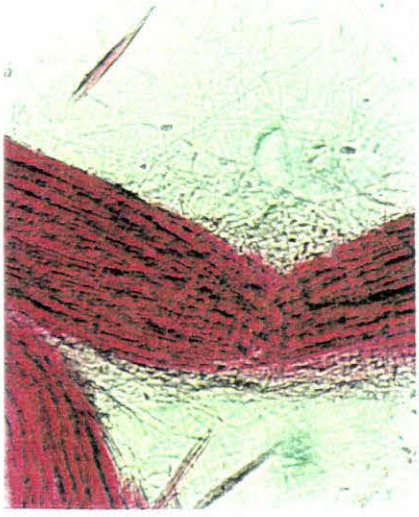


e

f

3.2.8 *Diheterospora chlamydosporium (8)*

Plate 3.2.8 a *D. chlamydosporium.* SDA, 21 days at 25°C.

Slow growing, raised cream to pale lemon thallus extending radially outward from scant centre. Colony had a cottony texture and emerged in a web-like pattern.

Plate 3.2.8 b *D. chlamydosporium.* Photomicroscopy (x400) above colony.

Aleuriospore state: Large, irregularly shaped, at first thin walled and hyaline, later becoming yellow/brown, very thick walled. Initiated as swollen terminal cells of short unbranched hyphae. These aleuriospores took up the Acid Fuschin stain quite readily. The accessory state (not illustrated) for the two strains involved in this study appeared to be *Verticillium* in type.

Plate 3.2.8 c *D. chlamydosporium.* BSW, shake culture, 21 days, 25°C.

A gelatinous mass encompassed the wool sample. There was a noticable reduction in cohesion of the wool sample over time.

Plate 3.2.8 d *D. chlamydosporium.* Photomicroscopy (x400) above colony.

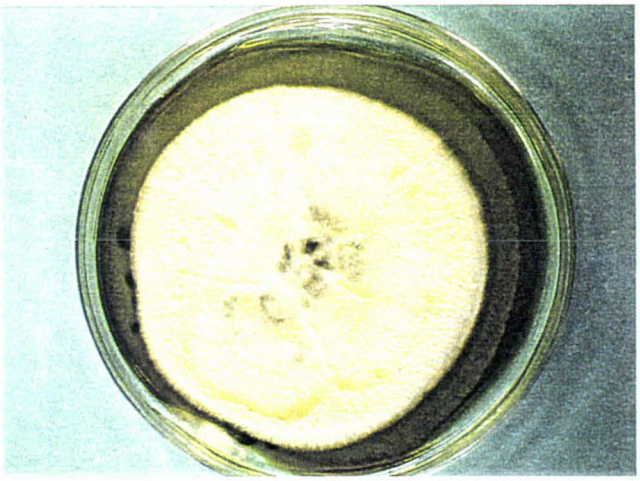
Fewer alueriospores were produced in this culture mode, but the overall morphology was the same as for Plate 3.2.8b.

Plate 3.2.8 e *D. chlamydosporium.* SDB, stationary culture, 21 days, 25°C.

This species showed very different growth morphology to the other species examined, with white, fluffy mycelial growth forming without much initial cohesion. This developed over time into scattered clumps.

Plate 3.2.8 f *D. chlamydosporium.* Photomicroscopy (x400) above colony

No apparent differences were seen microscopically for this culture compared to that grown on solid media.



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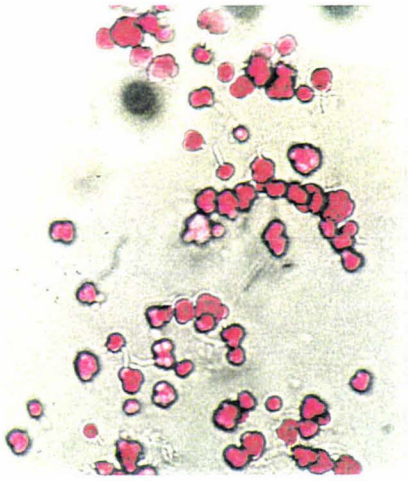

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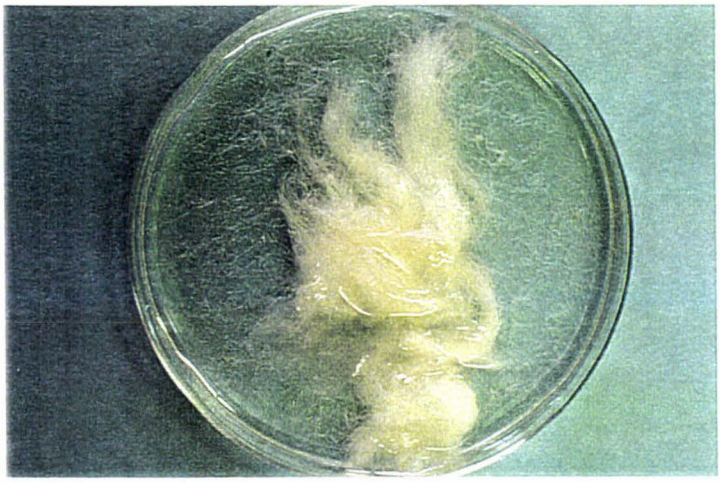

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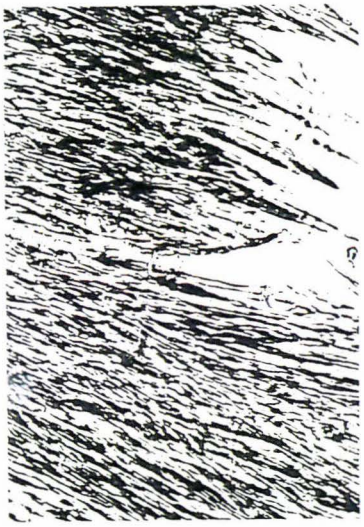

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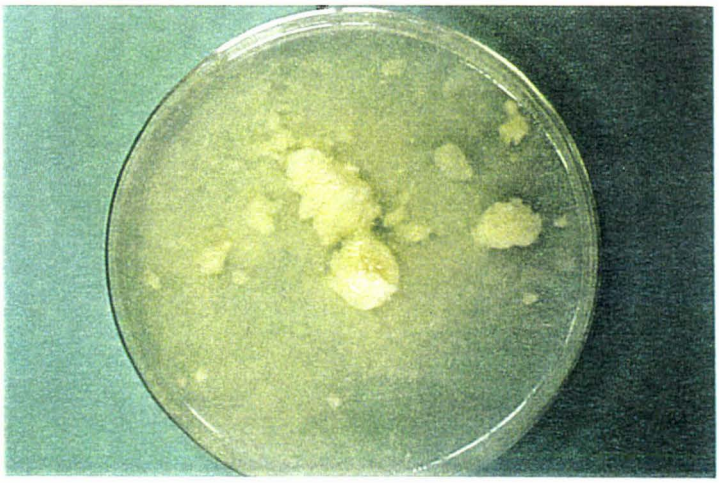

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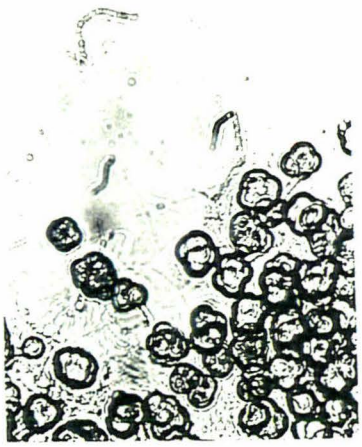

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3.3 Establishment of cultual techniques

3.3.1 Preliminary assessment of relative proteolytic activity

In early trials for assement of proteolytic activity (Results 3.4) observations of gross colony morphology showed that visible growth had not been as rapid or abundant by comparison to cultures of the same strains previously grown. Microscopic examination of the cultures showed that hyphal growth and extension was not as abundant as in previous cultures and there appeared also to be reduced spore development.

Among the *Microsporum spp. & Trichophyton mentagrophytes var mentagrophytes*, macrospore development, present during initial investigations, was non-existent and microspore development appeared less abundant.

The macrospores of *T. ajelloi* appeared to be reduced in number while in *S. brevicaulis* hyphal production and spore numbers also appeared reduced. In the *A. terreus* culture there was no spore development detected microscopically and no cliestothecal development seen.

Consequently, gelatin hydrolysis techniques (Methods 2.4.1 & 2.4.2) were used as indicators of relative extracellular proteolytic enzyme expression by isolates.

Initial results from the Charcoal gelatin disc test (table 3.4.1a) and also from the Gel hydrolysis plate test (table 3.4.2a) showed clearly that there was very little proteolytic enzyme production occurring during the incubation periods.

Enzyme activity detectable via the disc method or clearly visible zones of hydrolysis, had not occurred to the extent expected. Therefore, along with a reduced growth rate overall there had also been a reduced level of detectable proteolytic enzyme production.

It was thought that this may have been due to the extensive use of chloramphenicol and cycloheximide in the culture media as reductions in growth along with morphological anomalies are not unknown because of this. It was decided to attempt growth recovery using Lactrimel medium, as this is known to be useful for the production of macrospores and general hyphal growth (Baxter *et al.*, 1980).

On subculture of the strains on LMA, colony development was rapid with microscopic examination showing microspore production had increased significantly and macrospore production had resumed. Followinging the re-establishment of hyphal growth and spore production, the cultures were inoculated into lactrimel broth cultures, and, following 14 days incubation, a second series of tests were set up.

3.3.1.1 Charcoal Gelatin Discs

a) Test 1

Early experiments were carried out using cultures grown in SDBXX medium. The spore suspensions utilised had at that time been produced from cultures grown and stored on solid media incorporating the same antibiotics.

The results indicate a lack of prolific proteolytic enzyme production.

b) Test 2

Following subculture and incubation of the fungi on LMA plates without antibiotics, the cultures were inoculated into LMB and following 14 days incubation, a second series of gelatin disc tests were set up.

The results (table 3.4.1b), showed there had been a substantial increase in extracellular proteolytic enzyme production, with all species involved showing a marked increase in proteolytic activity.

Table 3.3.1a Charcoal Gelatin Discs test 1

(SDBXX culture filtrates)

KEY: - = Nil result

+ = Slight disintegration

+ + = Over ½ disc disintegrated O/N = overnight

+ + + = Total disc disintegration

Isolate	**Code**	**15min**	**30min**	**2hr**	**O/N**
M. canis	A	-	-	-	-
M. cookei	6	-	-	-	+
M. cookei	K	-	-	-	+
M. gypseum	B	-	-	-	+
M. nanum	L	-	-	-	-
T. mentagrophytes var. mentagrophytes	D	-	-	-	+
T. mentagrophytes var. erinacei	M	-	-	-	+
T. terrestre	J	-	-	-	+
T. ajelloi	E	-	-	-	-
S. brevicaulis	F	-	-	-	++
C. keratinophilum	I	-	-	-	-
A. terreus	H	-	-	-	-

Table 3.3.1b Charcoal Gelatin Discs test 2

(LMB culture filtrates)

KEY: - = Nil result

\+ = Slight disintegration

\+ + = Over ½ disc disintegrated O/N = overnight

\+ + + = Total disc disintegration

Isolate	**Code**		**15min**	**30min**	**2hr**	**O/N**
M. canis	A	-	+	++	+++	
M. cookei	6		-	-	++	+++
M. cookei	K	-	-	++	+++	
M. gypseum	B	-	-	++	+++	
M. nanum	L		-	-	+	+++
T. mentagrophytes var. mentagrophytes	D	-	+	++	+++	
T. mentagrophytes var. erinacei	M		-	-	++	+++
T. terrestre	J		-	+	+	+++
T. ajelloi	E		-	-	+	+++
S. brevicaulis	F	-	+	++	+++	
C. keratinophilum	I		-	-	++	+++
A. terreus	H	-	-	++	+++	

3.3.1.2 Gelatin Hydrolysis Plates

This method was used in conjunction with the gelatin disc method to screen for proteolytic enzyme production . Enzyme activity is detected as clearly visible zones of hydrolysis, radially extending away from the colony.

a)Test 1

These plates were inoculated from SDAXX cultures and the initial results mirrored those found using the gelatin disc method. In many cases the colony growth extended slightly past the zone of visible hydrolysis brought about by the degradation of the gelatin in the medium.

b) Test 2

Following growth on LMA, spore suspensions were inoculated into LMB and following 14 days incubation, inoculum from these cultures were used for a second series of tests.

Fewer species were selected and the results are as shown in table 3.3.1.2b There was an increase in visually detectable proteolytic activity, particularly in the case of the *M. cookei* strain and the *T. mentagrophytes var mentagrophytes* strain.

Table 3.3.1.2a Gelatin Hydrolysis Plates test 1

(From SDBXX cultures)

Isolate	**Code**	***Cc/cd**
M. canis	A	<1.00
M. cookei	6	<1.00
M. cookei	K	<1.00
M. gypseum	B	1.00
M. nanum	L	1.00
T. mentagrophytes var. mentagrophytes	D	1.00
T. mentagrophytes var.erinacei	M	1.00
T. ajelloi	E	<1.00
T terrestre	J	<1.00
S. brevicaulis	F	1.00
C. keratinophilum	I	<1.00
A. terreus	H	<1.00

Table 3.3.1.2b Gelatin Hydrolysis Plates test 2

(From LMB cultures)

Isolate	**Code**	***Cc/cd**
M. canis	A	1.30
M. cookei	K	2.30
M. gypseum	B	1.00
T. mentagrophytes var. mentagrophytes	D	1.75
T. ajelloi	E	1.30
S. brevicaulis	F	1.00
A. terreus	H	1.25
D. chlamydosporium.	8	1.70

*Cc = Radius of hydrolysis (cm); cd = colony radius (cm)

3.3.2 Spore counts

As was noted (Results 3.3.1), it was observed microscopically that spore formation, both macro and microspore,was sparse in all the strains being examined and proteinase production was much reduced. This was possibly due to the incorporation of cycloheximide and chloramphenicol in the culture media (See Results 3.4).

The strains were therefore cultured on LMA or dilSDA as "recovery media" and the number of spores present in all subsequent inocula determined to ensure there were sufficient to produce satisfactory growth.

The spore counts in the standard inocula (100µl) were estimated as in Methods 2.3.2. They were not adjusted to a particular level given that in nature the spores and growing hyphae are not "regulated". In theory, only one spore or segment of growing hypha is required for growth and proliferation to a visible colony.

The spore suspensions produced from LMA were used to inoculate the LMB cultures (Results 3.4), while spore suspensions produced from Dil.SDA were used to inoculate the SDB and later BSW cultures. (Results 3.4 and 3.5).

Table 3.3.2.1 Spore Counts for Standard Inocula from Lactrimel Agar Slope Cultures Used for LMB enzyme production cultures.

Species/strain	Code	Spores/ml
M. canis	A	4.78×10^4
M. gypseum	B	6.62×10^3
M. cookei	K	3.55×10^4
T. mentagrophytes var mentagrophytes	D	6.30×10^3
T. ajelloi	E	3.52×10^3
S. brevicaulis	F	3.90×10^5
A. terreus	H	3.64×10^4

Table 3.3.2.2 Spore Counts for Standard Inocula from Dil. SDA Agar Slope Cultures Used for BSW & SDB Enzyme production cultures.

Species/strain	Code	Spores/ml
M. canis	1	110.9×10^5
M. canis	2	169.0×10^5
M. canis	3	100.0×10^5
M. canis	A	109.8×10^5
M. cookei	4	287.7×10^5
M. cookei	5	149.4×10^5
M. cookei	6	262.6×10^5
M. cookei	C	379.7×10^5
M. cookei	K	82.2×10^5
M. gypseum	B	126.1×10^5
M. nanum	L	124.5×10^5
T. mentagrophytes var mentagrophytes	D	198.7×10^5
T. mentagrophytes var erinacei	M	202.2×10^6
T. ajelloi	E	19×10^5
T. terrestre	J	115.7×10^6
S. brevicaulis	F	54.8×10^5
A. terreus	H	133.3×10^5
C. keratinophylum	I	105.7×10^6
D.chlamydosporium	7	87.1×10^5
D. chlamydosporium	8	66.6×10^5

3.3.3 Measurement of pH vs Time

As well as ensuring sufficient spores were present in the inocula for medium used for enzyme production. Measurements of the pH of the broth culture changes with time were made.

Results of pH vs time in days are illustrated for representatives of species examined in liquid culture.

The LMB cultures were measured over 14 days and BSW cultures over 28 days as these were the culture periods used for examination of proteolytic enzyme production. Uninoculated culture media were included as controls. Measurements were made on the same day as a corresponding SDS-PAGE gel was run.

Illustrative results are presented here, but full measurements were recorded for all the strains cultured in various liquid media and examined by SDS-PAGE assay.

STRAINS ILLUSTRATED:

M. canis (A)

M. cookei (K)

M.gypseum (B)

T. mentagrophytes
var mentagrophytes (D)

S.brevicaulis (F)

A.terreus (H)

D. chlamydosporium (8)(809)

In the BSW cultures, the control medium showed a pH decrease in the first 7 days, followed by an increase to the original pH (Fig 3.3.3.1b), as no bacteria or fungi were isolated from the medium it was concluded that this was an artefact of the procedure.

General trends observed in the BSW cultures saw an initial reduction in the pH of the stationary cultures after 7 days incubation as in the controls, followed by an increase in pH over 7 to 28 days. In shake cultures however, the trend was towards an increase in the medium pH at 7 days sometimes followed by a decrease at 14 to 28 days.

In LMB cultures, a marked increase in pH occurred at 7 & 14 days in shake culture of most species. In stationary cultures, the initial growth delay was reflected in minimal change in pH at 7 d. except in *S. brevicaulis* and *D. chlamydosporium*, their pH rose.

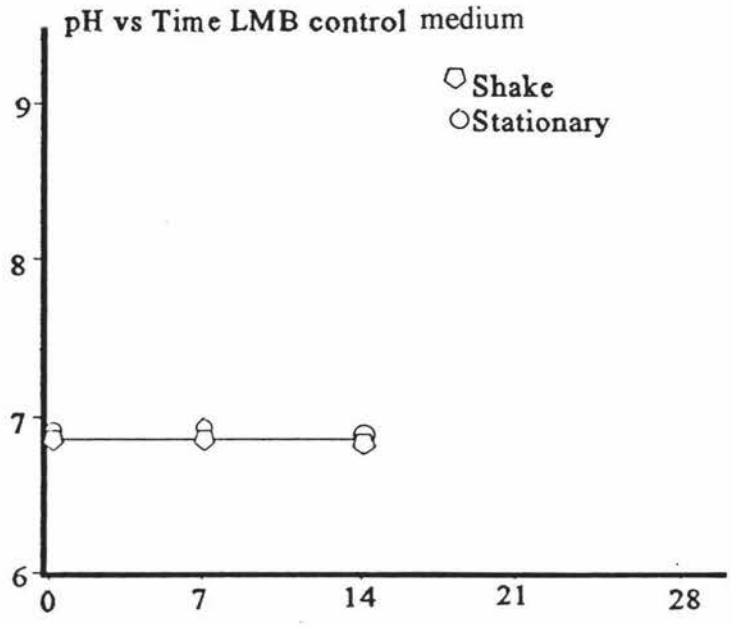
Fig 3.3.3.1a pH Vs Time LMB uninoculated control medium

This media showed no alteration of pH over the 14 day incubation period but remained constant at pH 6.8.

Fig 3.3.3.1b pH Vs Time BSW uninoculated control medium

In the first 7 days of incubation, the pH for both cultures fell from pH 8.2 to pH 7.8, then returned to pH 8.2 by day 14. This trend was consistent for all BSW cultures.

a


pH vs Time LMB control medium
Shake
Stationary
9
8
7
6
0
7
14
21
28
Time in Days

b

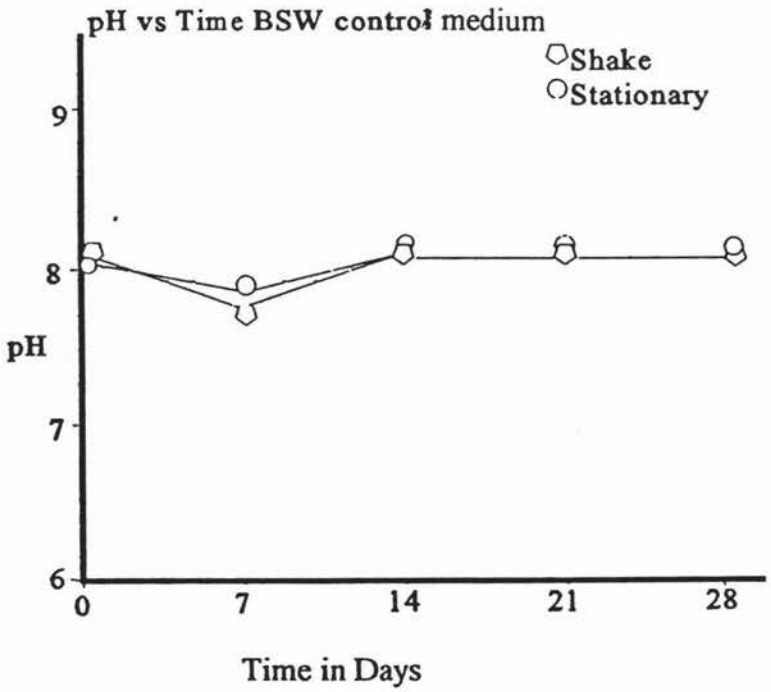

pH vs Time BSW control medium
Shake
Stationary
9
8
7
6
pH
0
7
14
21
28
Time in Days

Fig 3.3.3.2a pH Vs time for LMB inoculated with *M. canis.*

The shake culture increased in alkalinity going from pH 6.8 to 7.8 during the experimental period.

The stationary culture initially dropped from pH 6.8 to 6.4 before increasing to pH 7.8 by day 14.

Fig 3.3.3.2b pH Vs time for BSW inoculated with *M. canis.*

The shake culture medium pH increased steadily over the first 14 days, going from pH 8.2 to 8.6 then remained constant for the duration of the 28 day study.

The stationary culture medium pH decreased in the first 7 days from pH 8.2 to 7.6 followed by an increase over the next 21days with the final pH recorded for this strain at 28 days being pH 8.5.

Fig 3.3.3.3a pH Vs time for LMB inoculated with *M. cookei.*

The shake culture increased over 7 days from pH 6.8 to 8.2 followed by a decrease to pH 7.6 by day 14.

The stationary culture however, showed no change in the first 7 days followed by an increase from pH 6.8 to 7.8 by day 14.

Fig 3.3.3.3b pH Vs time for BSW inoculated with *M. cookei.*

The shake culture showed an increase from pH 8.2 to pH 8.5 in the first 7 days, reaching pH 8.6 by day 14, this was followed by a decrease to pH 8.3 by day 21 where it remained stable for the remainder of the trial period.

The stationary culture showed little change over the 28 day period.

Fig 3.3.3.4a pH Vs time for LMB inoculated with *M. gypseum.*

The shake culture increased from pH 6.8 to 8.8 over the 14 days.

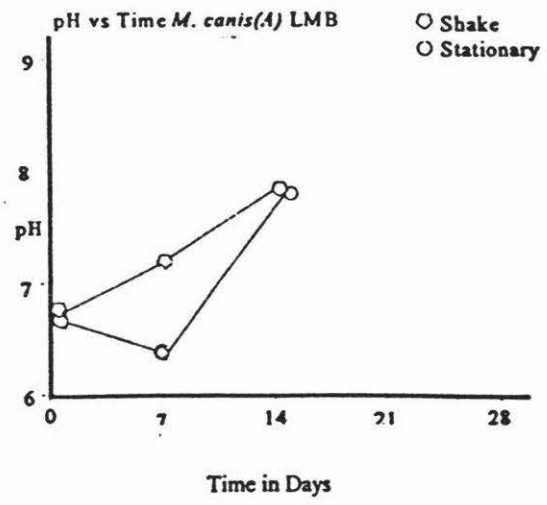
The stationary culture initially decreased from pH 6.8 to 6.5 followed by an increase to pH 7.2 by day 14.

Fig 3.3.3.4b pH Vs time for BSW inoculated with *M. gypseum.*

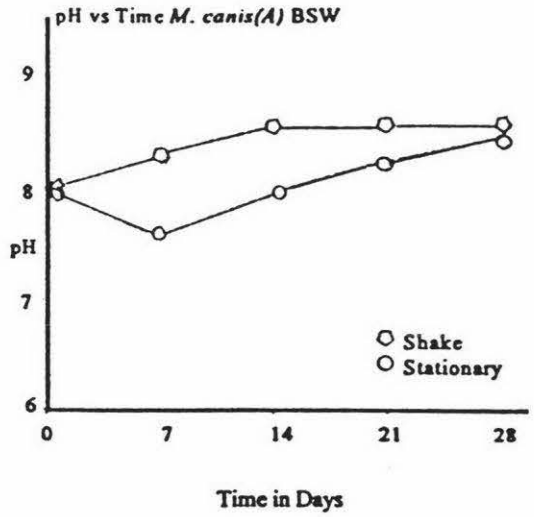
The shake culture pH increased in the first 14 days from pH 8.2 to 8.5 and did not increase further over the remainder of the trial period.

The stationary culture initially decreased in pH to pH 7.5, but this was followed by an increase and at 21 days the pH stabilised at pH 8.4.

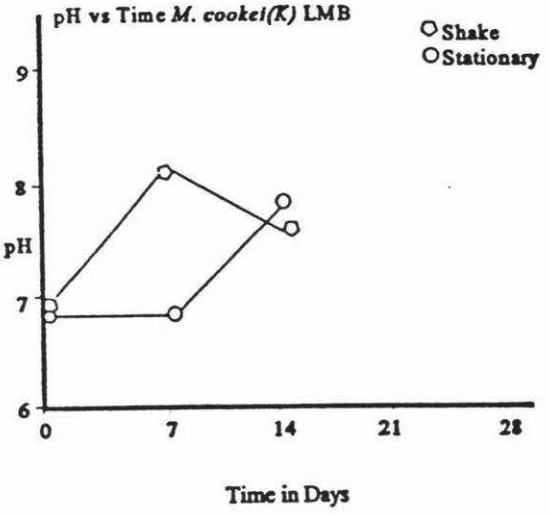
a


pH vs Time M. canis(A) LMB
Shake
Stationary
9
8
pH
7
6
0
7
14
21
28
Time in Days

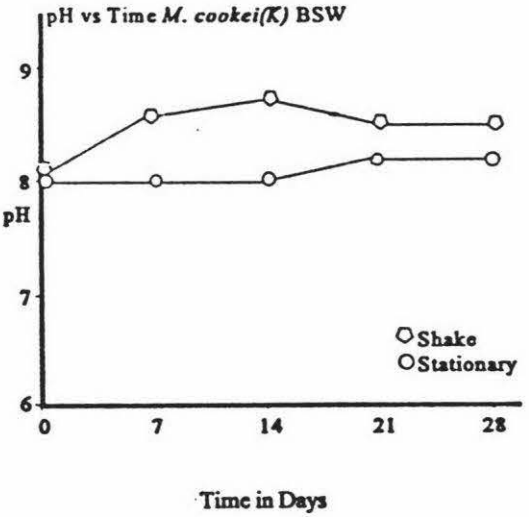
b


pH vs Time M. canis(A) BSW
9
8
pH
7
6
Shake
Stationary
0
7
14
21
28
Time in Days

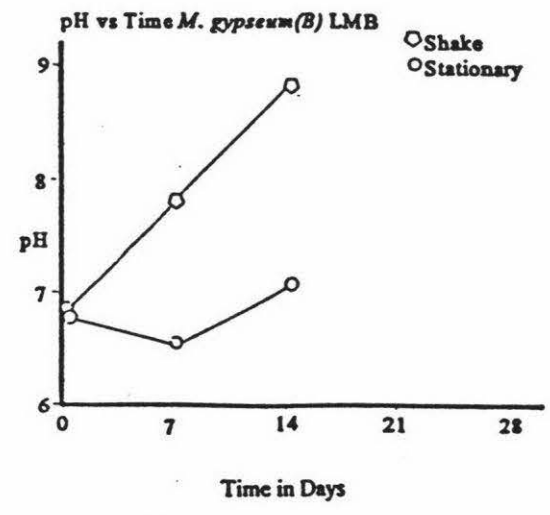
a


pH vs Time M. cookei(K) LMB
Shake
Stationary
9
8
pH
7
6
0
7
14
21
28
Time in Days

b


pH vs Time M. cookei(K) BSW
9
8
pH
7
6
Shake
Stationary
0
7
14
21
28
Time in Days

a


pH vs Time M. gypseum(B) LMB
Shake
Stationary
9
8
pH
7
6
0
7
14
21
28
Time in Days

b

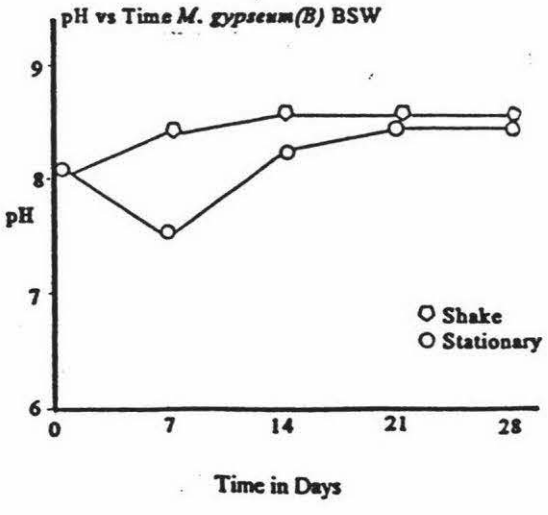

pH vs Time M. gypseum(B) BSW
9
8
pH
7
6
Shake
Stationary
0
7
14
21
28
Time in Days

Fig 3.3.3.5a pH Vs time for LMB inoculated with *T. mentagrophytes*

The shake culture showed an increase in pH over the 14 days moving from pH 6.8 to pH 8.2 in the first 7 days then to pH 8.7 by day 14.

The stationary culture showed a slight decrease from pH 6.8 to 6.6 in the first 7 days followed by an increase to pH 7.6 by day 14.

Fig 3.3.3.5b pH Vs time for BSW inoculated with *T. mentagrophytes.*

The shake culture showed a steady increase over the 28 day period moving from pH 8.2 to pH 8.8.

The stationary culture showed an initial drop from pH 8.2 to 7.8 in the first 7 days followed by a steady increase over the trial period with the final pH 8.7.

Fig 3.3.3.6a pH Vs time for LMB inoculated with *T. ajelloi.*

The shake culture showed an increase from pH 6.8 to 8.0 in the first 7 days followed by an increase to pH 8.4 by day 14.

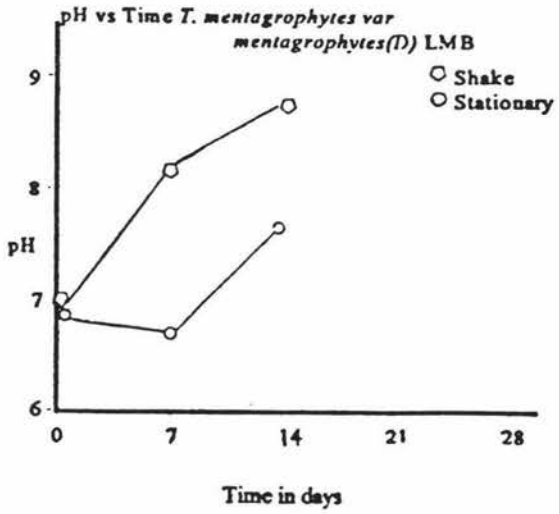
The stationary culture showed a decrease to pH 6.7 at 7 days followed by an increase to pH 7.15 by day 14.

Fig 3.3.3.6b pH Vs time for BSW inoculated with *T. ajelloi.*

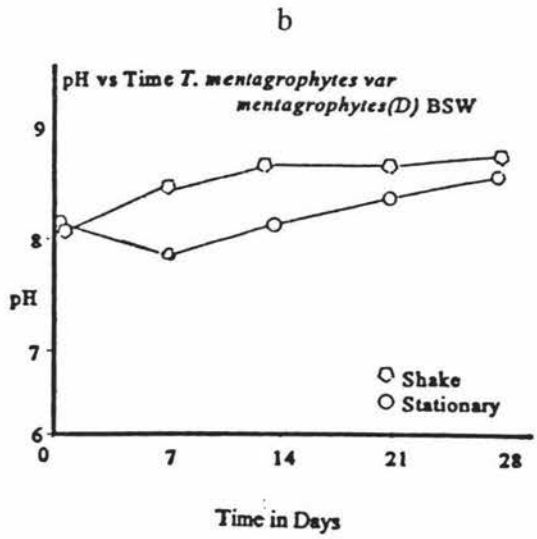
The shake culture showed an increase over 21 days from pH 8.2 to pH 8.7 following which a decrease to pH 8.6 was noted at day 28.

The stationary culture showed a decrease at 7 days from pH 8.2 to 7.7 followed by an increase to pH 8.2 by day 28.

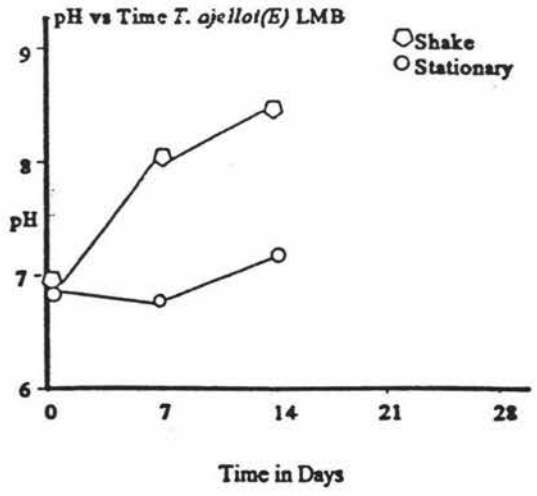
a


pH vs Time T. mentagrophytes var mentagrophytes(D) LMB
Shake
Stationary
9
8
7
6
pH
0
7
14
21
28
Time in days

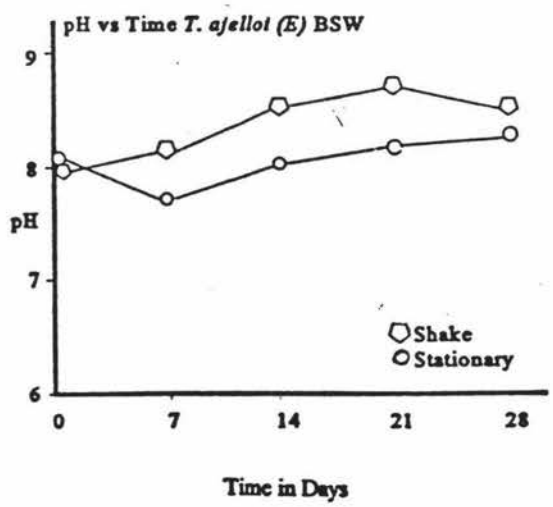
b


pH vs Time T. mentagrophytes var mentagrophytes(D) BSW
9
8
7
6
pH
Shake
Stationary
0
7
14
21
28
Time in Days

a


pH vs Time T. ajellot(E) LMB
Shake
Stationary
9
8
7
6
pH
0
7
14
21
28
Time in Days

b


pH vs Time T. ajellot (E) BSW
9
8
7
6
pH
Shake
Stationary
0
7
14
21
28
Time in Days

3.3.3.7a pH Vs time for LMB inoculated with *S. brevicaulis.*

The shake culture showed an increase over the 14 days from pH 6.8 to 8.8.

The stationary culture showed a decrease over the 14 days from pH6.8 to 5.6.

3.3.3.7b pH Vs time for BSW inoculated with *S. brevicaulis.*

The shake culture showed an initial increase from pH 8.2 to 8.6 in the first 7 days and stabilised there for the duration of the trial period.

The stationary culture showed an initial drop from pH 8.2 to 7.8 followed by an increase over the trial period to pH 8.4.

3.3.3.8a pH Vs time for LMB inoculated with *A. terreus.*

The shake culture intially showed a slight increase from pH 6.8 to 7.0, but this was followed by a substantial decrease to pH 5.6 by day 14.

The stationary culture showed an initial decrease to pH 6.5 followed by a substantial increase to pH 7.7 by day 14.

3.3.3.8b pH Vs time for BSW inoculated with *A. terreus.*

The shake culture showed a steady increase in the first 14 days to pH 8.7 followed by a decrease in pH by day 21, this then was followed by an increase back to pH 8.7 by day 28.

The stationary culture showed an initial decrease followed by a steady increase to pH 8.3 by 28 days.

3.3.3.9a pH Vs time for LMB inoculated with *D. chlamydosporium* (8)

The shake culture showed an initial substanial decrease from pH 6.8 to 5.5 followed by an equally substantial increase to pH 7.8 by day 14.

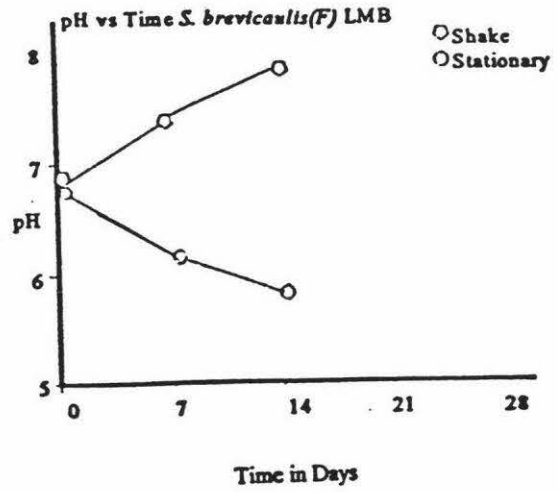
The stationary culture, showed only a small drop to pH 7.4 at day 7 followed by an increase to pH 7.7 by day 14.

3.3.3.9b pH Vs time for BSW inoculated with *D. chlamydosporium* (8)

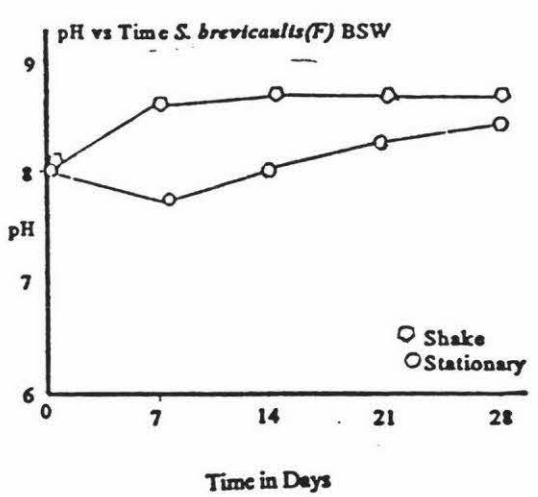
The shake culture showed an initial increase to pH 8.6 over 14 days followed by a decrease over the next 14 days to pH 8.4.

The stationary culture showed an initial decrease to pH 7.6 at 7 days followed by an increase over the next 21 days to pH 8.3.

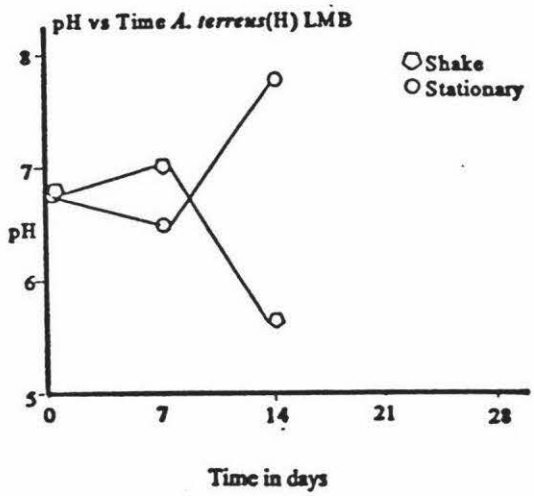
a


pH vs Time S. brevicaulis(F) LMB
Shake
Stationary
pH
8
7
6
5
0
7
14
21
28
Time in Days

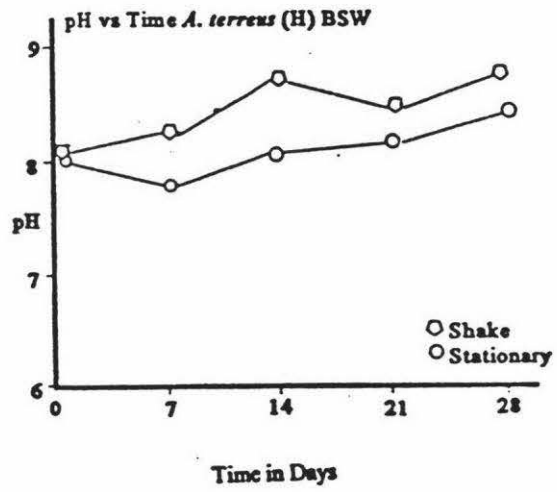
b


pH vs Time S. brevicaulis(F) BSW
pH
9
8
7
6
Shake
Stationary
0
7
14
21
28
Time in Days

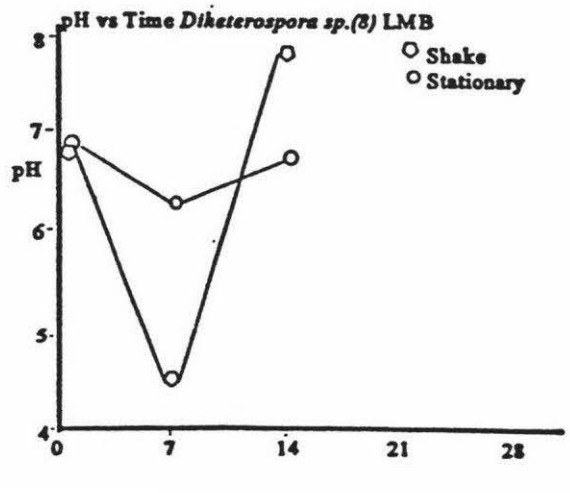
a


pH vs Time A. terreus(H) LMB
Shake
Stationary
pH
8
7
6
5
0
7
14
21
28
Time in days

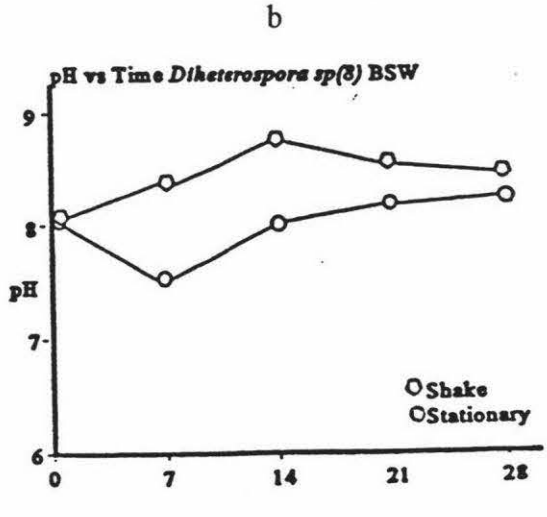
b


pH vs Time A. terreus (H) BSW
pH
9
8
7
6
Shake
Stationary
0
7
14
21
28
Time in Days

a


pH vs Time Diheterospora sp.(8) LMB
Shake
Stationary
pH
8
7
6
5
4
0
7
14
21
28
Time in Days

b


pH vs Time Diheterospora sp(8) BSW
pH
9
8
7
6
Shake
Stationary
0
7
14
21
28
Time in Days

3.4. Assessments of gelatinase production in broth cultures

Culture filtrates were prepared as in Methods 2.6.3.2 and proteinase expression as gelatinases was compared by the substrate co-polymerised SDS-PAGE technique (Methods 2.6.2).

As has been noted, fungi which had been cultured on SDAXX and in SDBXX stationary culture were examined with disappointing results(Column 1 figs 3.4.1-3.4.5). Following this various strains which had been utilised by previous workers in this laboratory were introduced. These strains had not been subjected to growth conditions incorporating antibiotics.

The strains selected from the culture collection were cultured initially in SDB shake cultures to ensure that they were still capable of producing proteolytic enzymes. This also allowed an assessment of the reproducibility of the technique between investigators. They were then cultured and assayed alongside the freshly isolated strains utilising LMB. (Columns 2 and 3 Figs 3.4.1,.3.4.2,3.4.5)

These culture assays allowed an assessment of the use of lactrimel broth as a suitable aid in the re-establishment of proteolytic enzyme production to levels detectable by the SDS-PAGE technique, they also demonstrated that the bands produced by the 'recovered' strains were consistent with those produced by strains not subjected to long term antibiotic use.

Illustrated are full results gained using strains of *M. canis* and *M. cookei*, as these species had been investigated by previous workers in this laboratory and always produced positive results. LMB cultures of *T. mentagrophytes var. mentagrophytes*, *S. brevicaulis* and the *D. chlamydosporum* isolates are also included.

Other cultures were also examined in these media but the results are not illustrated here; banding patterns were inconsistent.

LEGEND:

7, 14, 21, 28	Incubation time in days (d)
LMB	Lactrimel broth medium
SDB	Sabourauds Broth Medium
SDBXX	Sabourauds Broth Medium incorporating chloramphenicol and cycloheximide
SH	Shake culture mode
ST	Stationary culture mode

Fig 3.4.1 Gelatinases of *M.canis* 1,2,3 & A

SDBXX Strain A, ST culture.

In stationary cultures inoculated with *M. canis (A)* only one band (65 Kda) was detected at 7 and 14 days.

SDB Strains 1,2 & 3, SH culture

At 7 days , three bands were clearly seen (85, 33 and 27 Kda) for all three strains. At 14 days, these bands were again detected together with an extra band at 52 Kda. At 21 days three bands were seen (52, 36 and 27 Kda). Strain "2" showed a large amount of smearing. At 28 days, any larger bands were indistinct due to smearing through the lanes, but three bands were seen for all three strains (33, 27 and 14 Kda).

LMB Strains 1, 3 & A

Strains 1,3 & A were selected for further investigations with LMB.

In the shake culture, at 7 days eight bands were seen (200, 145, 105, 50, 26, 25, 21 and 11 Kda). At 14 days these bands were again seen, with several bands (145, 105, and 11 Kda) being more pronounced suggesting an increased production of these enzymes.

In stationary cultures at 7 days, eight bands were seen (214, 135, 80, 63, 37, 34, 26 and 16 Kda) At 14 days, extensive gelatinolysis was present in the upper portions of the lanes but six bands were seen in all three strains (98, 80, 37, 30, 25 and 12 Kda).

Band production was increased 7x in LMB ST cultures compared to SDBXX ST cultures and there was consistency between different strains, although the banding pattern was different in stationary and shake cultures.

1 2 3 4

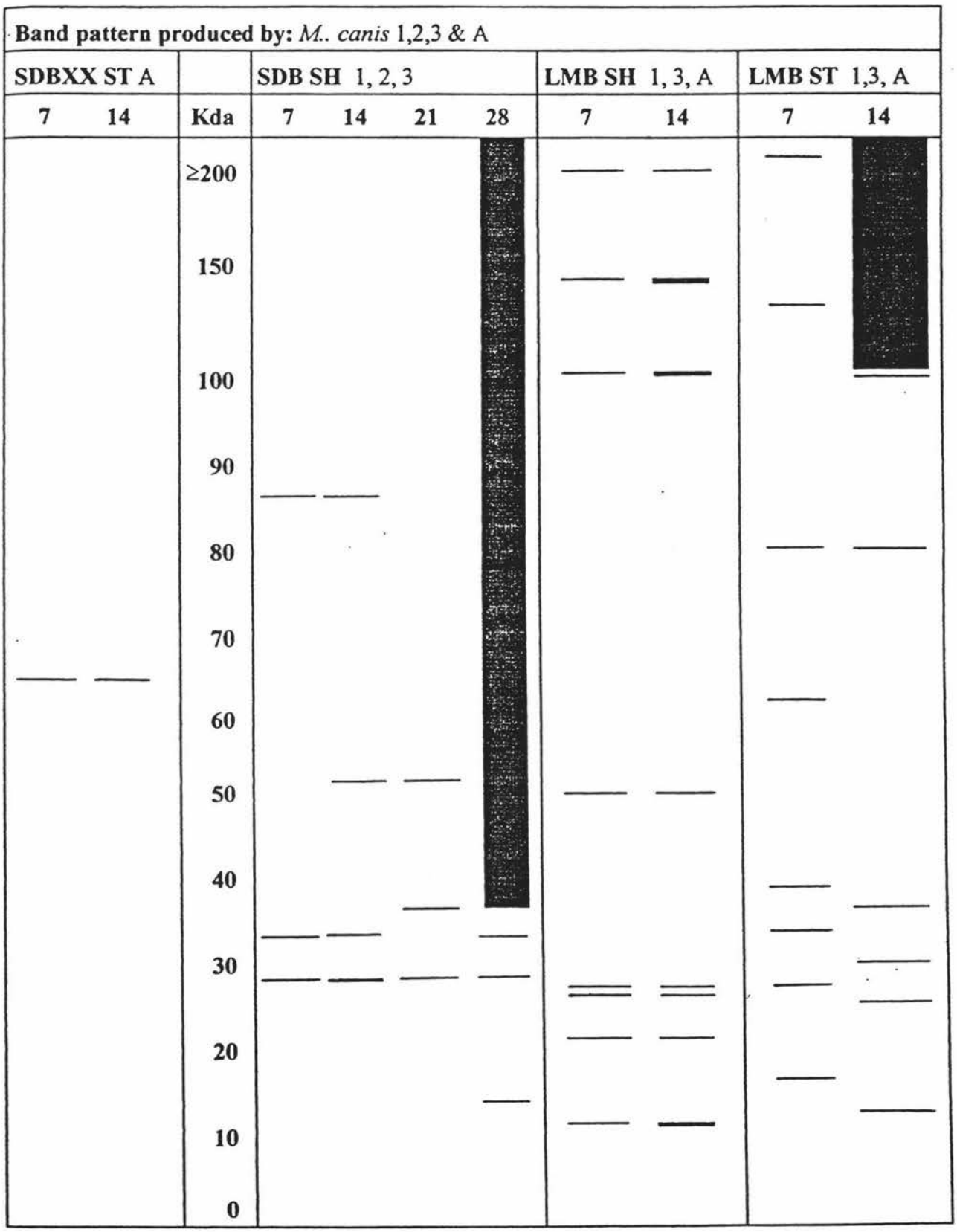

Band pattern produced by: M.. canis 1,2,3 & A
SDBXX ST A
SDB SH 1, 2, 3
LMB SH 1, 3, A
LMB ST 1,3, A
7
14
Kda
7
14
21
28
7
14
7
14
≥200
150
100
90
80
70
60
50
40
30
20
10
0

Fig 3.4.2 Gelatinases of *M. cookei* 6 & K

SDBXX Strain K, ST culture

No bands were present for this strain and only a very faint smear of gelatinolysis was seen in the lane running this culture filtrate.

SDB Strains 4,5 & 6, SH culture

At 7 days incubation three bands were seen (89, 23 and 20 Kda), for all three strains. At 14 days three bands were seen (89, 40, and 23 Kda) for strains 4 & 6; in strain 5 a 26 Kda band was also present. At 21 days, a great deal of smearing was found in the lanes with only the smaller bands (23 and 20 Kda) detected, while at 28 days bands were detected at 100, 75, 25 and 23 Kda (strain 4), 47 and 19 Kda (strain 5) and 102, 23, 22 and 16 Kda (strain 6).

LMB Strains 6 & K

Strains 6 and K were selected for further culture and investigations.

In shake cultures, after 7 days incubation, four bands were produced by both strains (145, 25, 22 and 19 Kda). But at 14 days variation in band sizes was observed between the two cultures with strain 6 showing three bands (145, 22 and 21 Kda), while in strain K, five bands were seen (145, 29, 23, 18 and 17 Kda).

In the stationary cultures, at 7 days five bands were detected in both cultures (>200, 135, 30, 25 and 22 Kda), at 14 days seven bands were seem in both cultures (138, 107, 25, 23, 22, 20 and 13 Kda).

In both cultures, several bands were consistently detected e.g. 145 Kda and 22 Kda. However, the 21 Kda band was confined to strain 6 while the 29, 18 and 17 Kda bands were confined to strain K.

Band production in LMB medium was more readily discernible than in SDB and some consistency of production was seen in terms of band sizes, but not in terms of total bands produced.

Band pattern produced by: *M. cookei* 4,5, 6 & K

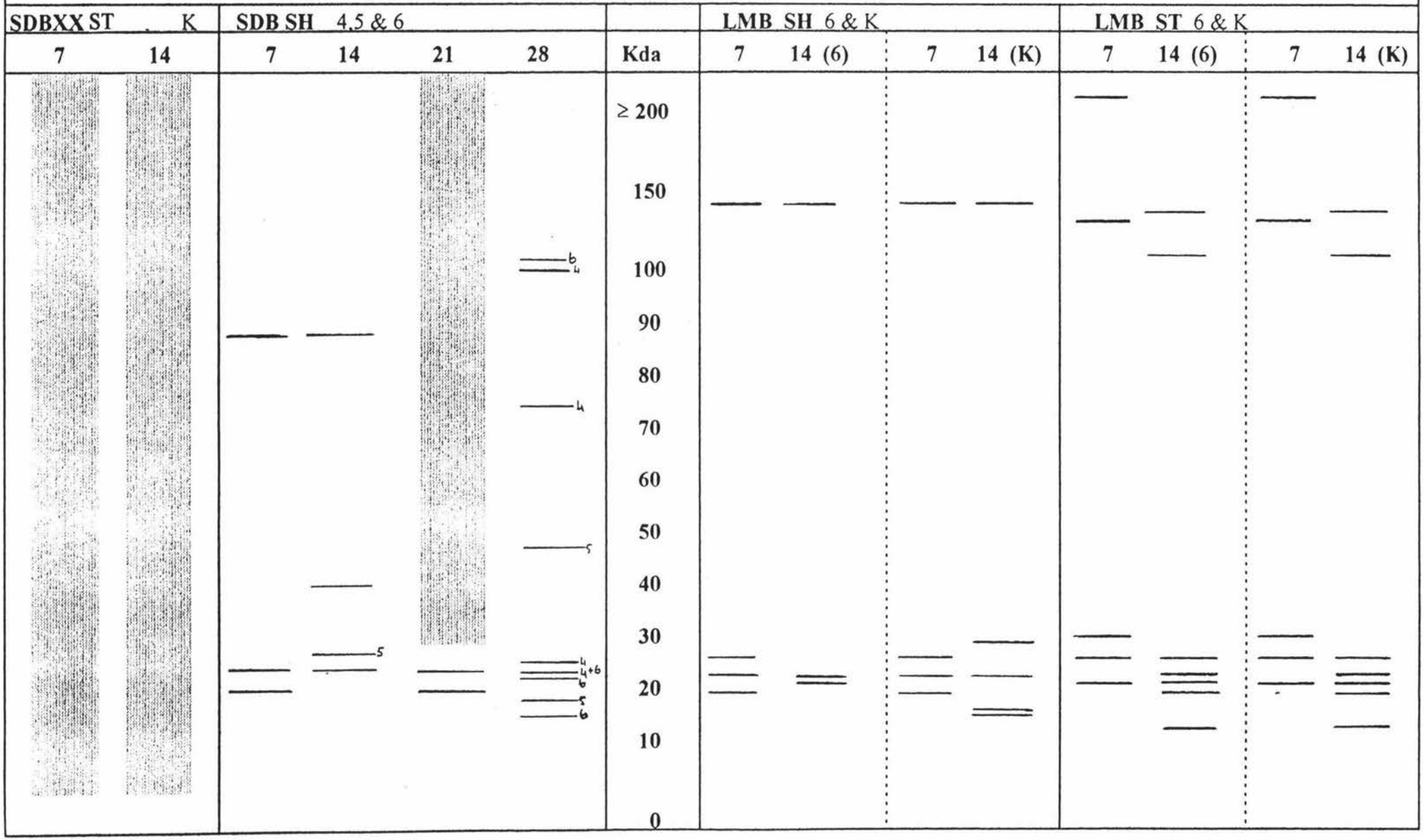


Fig 3.4.3 Gelatinases of *T. mentagrophytes var. mentagrophytes* D

SDBXX ST culture

In stationary cultures at 7 days five bands were seen (70, 40, 17, 13 and 10 Kda) and at 14 days the same bands were present except for the 13 Kda.

LMB

In shake culture at 7 days, four bands were seen (200, 191, 145 and 25 Kda). At 14 days, extensive gelatinolysis occurred in the upper portion of the gel. Those bands seen were at 68, 21 and 7 KDa. There was no consistency of band production seen with this culture.

In stationary culture at 7 days, four proteolytic bands were seen (118, 87, 30 and 17 Kda). At 14 days,as in the shake culture an extensive area in the upper portion of the lane had been gelatinised. However, eight bands were seen (63, 37, 32, 30, 22, 19, 17 and 8 Kda).

The bands found at 30 and 17 Kda appeared to be produced consistently over the 14 day period.

For this strain, no increase in band production was seen in LMB ST cultures compared to SDBXX. While no consistency of band production in the two sampling periods was seen in shake cultures for LMB medium, there was some consistency seen in stationary cultures.

1 2 3

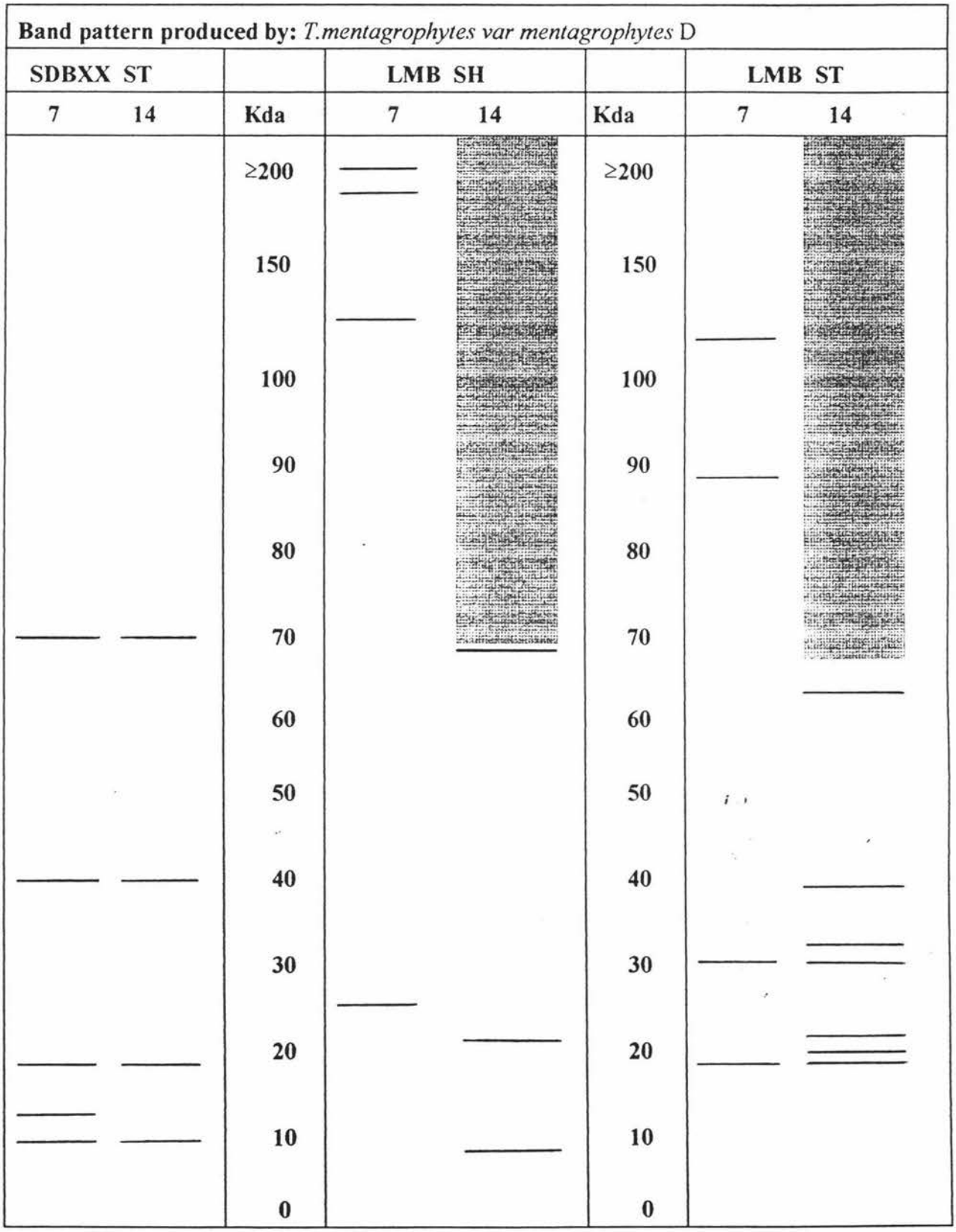

Band pattern produced by: T.mentagrophytes var mentagrophytes D
SDBXX ST
LMB SH
LMB ST
7
14
Kda
≥200
150
100
90
80
70
60
50
40
30
20
10
0

Fig 3.4.4 Gelatinases of *S. brevicaulis* F

SDBXX ST culture

Following 7 days incubation, two bands were seen (75 and 10 Kda).
At 14 days, again two bands were seen (65 and 10 Kda). These bands were well outlined and defined even though there were large areas of gelatinolysis leading to them which may have obscured other bands.

LMB

In shake culture following 7 days incubation extensive gelatinolysis was consistently found in the upper portion of lanes loaded with this filtrate.
At 7 days eight bands were seen (100, 72, 35, 23, 17, 12, 9 and 8 Kda); and 14 days ten bands (100, 82, 72, 35, 27, 23, 18, 17, 12 and 8 Kda). Thus seven of the bands were consistently demonstrated over the 14 day trial period

In the stationary culture following 7 days incubation ten bands were seen (117, 76, 40, 32, 27, 25, 22, 18, 13 and 10 Kda). At 14 days there was extensive gelatinolysis in the upper portion of the lane but bands were seen at 28,24, 23, 22 and 7 Kda.

1 2 3

Band pattern produced by: *S. brevicaulis* F

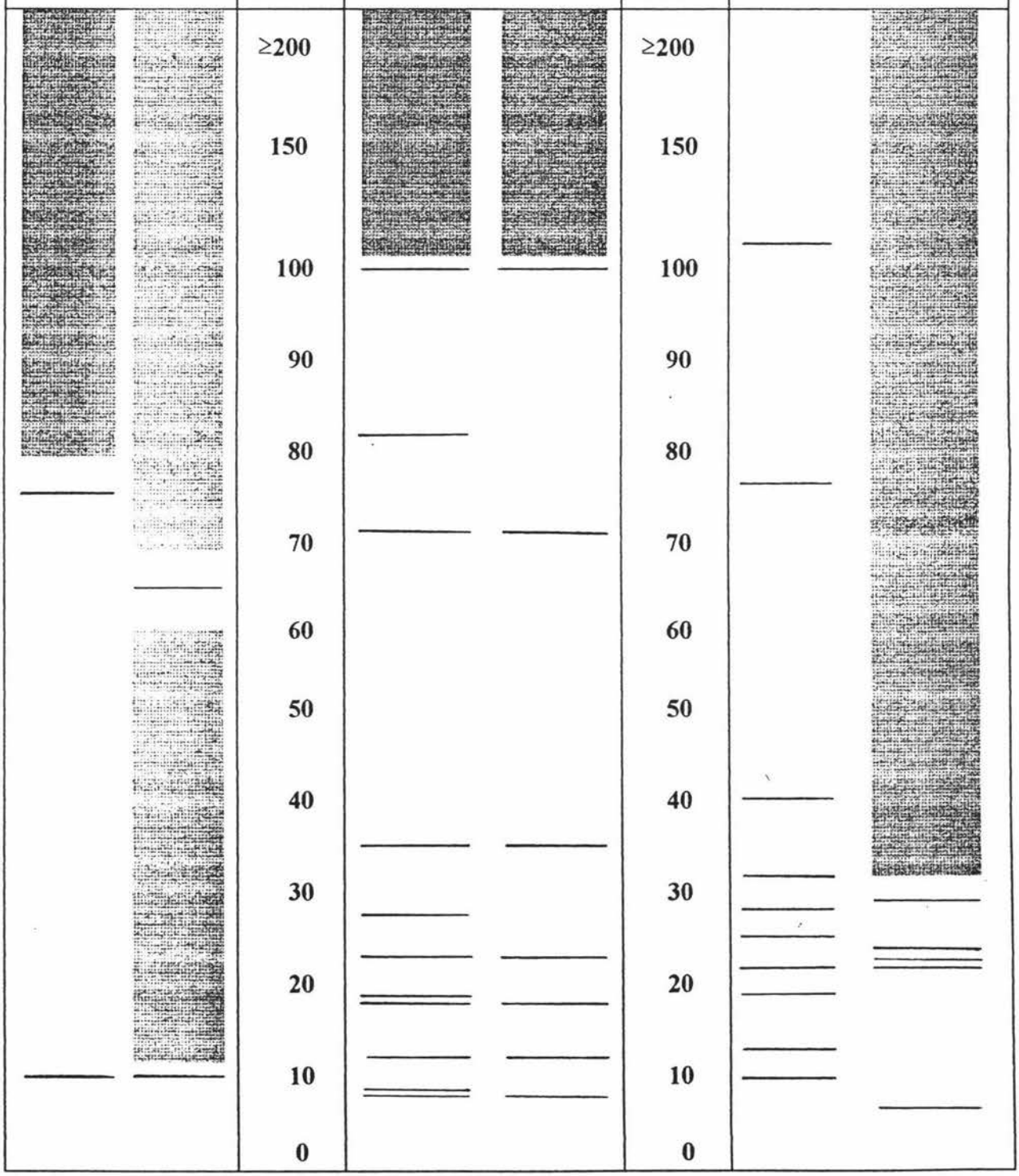


Fig 3.4.5 Gelatinases of *D. chlamydosporium (7 & 8)*

These strains were not examined in SDBXX.

SDB SH cultures

At 7 days four bands were seen (182, 40, 15 and 12 Kda), at 14 days five bands were seen (170, 107, 48, 24 & 21 Kda). At 21 days one band was detected at 70 Kda for strain 7 and two bands (20 & 18 Kda) for strain 8. At 28 days two bands were seen (70 and 24 Kda) for both strains along with extensive gelatinolysis in the lanes containing these culture filtrates.

Gelatinases of *D. chlamydosporium (7)*

LMB

In shake culture following 7 days incubation four bands were seen (200, 191, 145 and 25 Kda). At 14 days, four different bands were detected (80, 60, 21 and 8 Kda).

In stationary culture following 7 days incubation four bands were present (214, 110, 38 and 19 Kda), while at 14 days seven bands were seen (138, 68, 28,24, 23,22 and 9 Kda). Thus no consistency of band production was seen for this strain in LMB medium.

Gelatinases of *D. chlamydosporium (8)*

LMB

In shake culture following 7 days incubation five bands were seen (200, 191, 145,15 and 11Kda), and at14 days five (70, 23, 13, 9 and 8 Kda).

In stationary culture following 7 days incubation six bands were present (214, 158, 35, 28, 20 and 19 Kda), and at 14 days seven (68, 28, 25, 23, 22, 17 and 9 Kda). In this culture, only the band detected at 28 Kda was seen in both gel runs, otherwise, no consistency of band production was seen.

1 2 3

Band pattern produced by: *Diheterospora sp.* 7

SDB SH					LMB SH			LMB ST	
7	14	21	28	Kda	7	14	Kda	7	14
				≥200			≥200		
				150			150		
				100			100		
				90			90		
				80			80		
				70			70		
				60			60		
				50			50		
				40			40		
				30			30		
				20			20		
				10			10		
				0			0		

1 2 3

Band pattern produced by: *Diheterospora sp.* 8

SDB SH					LMB SH			LMB ST	
7	14	21	28	Kda	7	14	Kda	7	14
				≥200			≥200		
				150			150		
				100			100		
				90			90		
				80			80		
				70			70		
				60			60		
				50			50		
				40			40		
				30			30		
				20			20		
				10			10		
				0			0		

Table 3.4.1 Band sizes for non-illustrated species examined SDBXX/LMB

Due to the large number of species being examined, it was decided not to illustrate all results. The band sizes detected are presented here in table form.

Bands Produced by: *M.gypseum* **Strain:** B

Media	Mode	Day	Kda >150	Kda 100-150	Kda 50-100	Kda 20-50	Kda < 20	Total Bands
SDBXX	ST	7			50			1
		14						0
						Total	Bands	1
LMB	Sh	7		115,	69	46,29,22	15	6
		14		115	69	46,29,22 21		6
						Total	Bands	12
	St	7		135	87,72, 66	43,27,25		7
		14	200	126	87,26, 25	12		6
						Total	Bands	13

Bands Produced by: *M. nanum* **Strain:** L

Media	Mode	Day	Kda >150	Kda 100-150	Kda 50-100	Kda 20-50	Kda < 20	Total Bands
SDBXX	St	7				45,37		2
		14				45,37		2
						Total	Bands	4
LMB	Sh	7		145		30,25, 24		4
		14		145		30,26, 25,24		5
						Total	Bands	9
LMB	St	7		148	79	37,34, 29	16	6
		14			69	39,27, 25,24	12	6
						Total	Bands	12

Table 3.4.1 cont.,

Bands Produced by: *T. ajelloi* **Strain:** E

Media	Mode	Day	Kda >150	Kda 100-150	Kda 50-100	Kda 20-50	Kda < 20	Total Bands
SDBXX	St	7	150	120				2
		14	150	120				2
						Total	Bands	4
LMB	Sh	7		105	80		18	3
		14		120,105	80,76		7	5
						Total	Bands	8
LMB	St	7	166	148			17	3
		14		115	71			2
						Total	Bands	5

Bands Produced by: *T. mentagrophytes var erinacei* **Strain:** M

Media	Mode	Day	Kda >150	Kda 100-150	Kda 50-100	Kda 20-50	Kda < 20	Total Bands
SDBXX	St	7		150,100			17	3
		14		120		20		2
						Total	Bands	5
LMB	Sh	7			96,76,66	28,23		5
		14			80,66	45,23	8	5
						Total	Bands	10
LMB	St	7			98	40	18,15	4
		14			52	26,25, 24,23	9	6
						Total	Bands	10

Bands Produced by: *T. terrestre* **Strain:** J

Media	Mode	Day	Kda >150	Kda 100-150	Kda 50-100	Kda 20-50	Kda < 20	Total Bands
SDBXX	St	7						0
		14						0
						Total	Bands	0
LMB	Sh	7		145	91,83	39,36		5
		14		145	73		7	3
						Total	Bands	8
LMB	St	7	240	135	100,72, 70			5
		14		107	78,68	24		4
						Total	Bands	9

Table 3.4.1 cont.

Bands Produced by: *A. terreus* **Strain:** H

Media	Mode	Day	Kda >150	Kda 100-150	Kda 50-100	Kda 20-50	Kda < 20	Total Bands
SDBXX	St	7	200					1
		14						0
						Total	Bands	1
LMB	Sh	7			95	38,35, 26		4
		14		110		26,21	16,9,8	6
						Total	Bands	10
LMB	St	7	166		59	45,40, 35,30	20,19	8
		14			71	28,25, 22	19,10	6
						Total	Bands	14

Bands Produced by: *C. keratinophilum* **Strain:** I

Media	Mode	Day	Kda >150	Kda 100-150	Kda 50-100	Kda 20-50	Kda < 20	Total Bands
SDBXX	St	7						0
		14						0
						Total	Bands	0
LMB	Sh	7			83	23		2
		14	200,166					2
						Total	Bands	4
LMB	St	7	213,160			46		3
		14						0
						Total	Bands	3

3.5 Gelatinase production in wool-containing media

Cultures in which the wool portion of the medium had undergone both ethylene oxide sterilisation and autoclaving and cultures in which the wool portion of the medium had undergone ethylene oxide sterilisation only were established with shake and stationary modes for each medium set up at regular intervals. Samples from each culture were taken at 7 day intervals and analysed by the substrate co-polymerised SDS-PAGE technique (Methods 2.5).

Due to the numbers of bands found in these media, actual band sizes for all BSW SDS-PAGE investigations are given in Appendix A.

LEGEND:

7, 14, 21, 28	Time in days (d)
EOBSW	Basal salts + wool broth medium, ethylene oxide sterilised only
AEOBSW	Basal salts + wool medium, autoclaved and ethylene oxide sterilised
SH	Shake culture
ST	Stationary culture
96	Cultures run in 1996
97	Cultures run in 1997

3.5.1 Gelatinases of *M. canis* (1,3 & A)

AEOBSW

In shake culture mode 33 % of bands seen were between 50 & 100 Kda and a further 29% were seen between 20 & 50 Kda. In stationary culture the majority of band production was in the <50 Kda range with these bands making up 56% of bands seen.

Bands at 151, 85, 25, 23 & 21 Kda were frequently seen in both culture modes during the incubation periods for this medium.

EOBSW 96

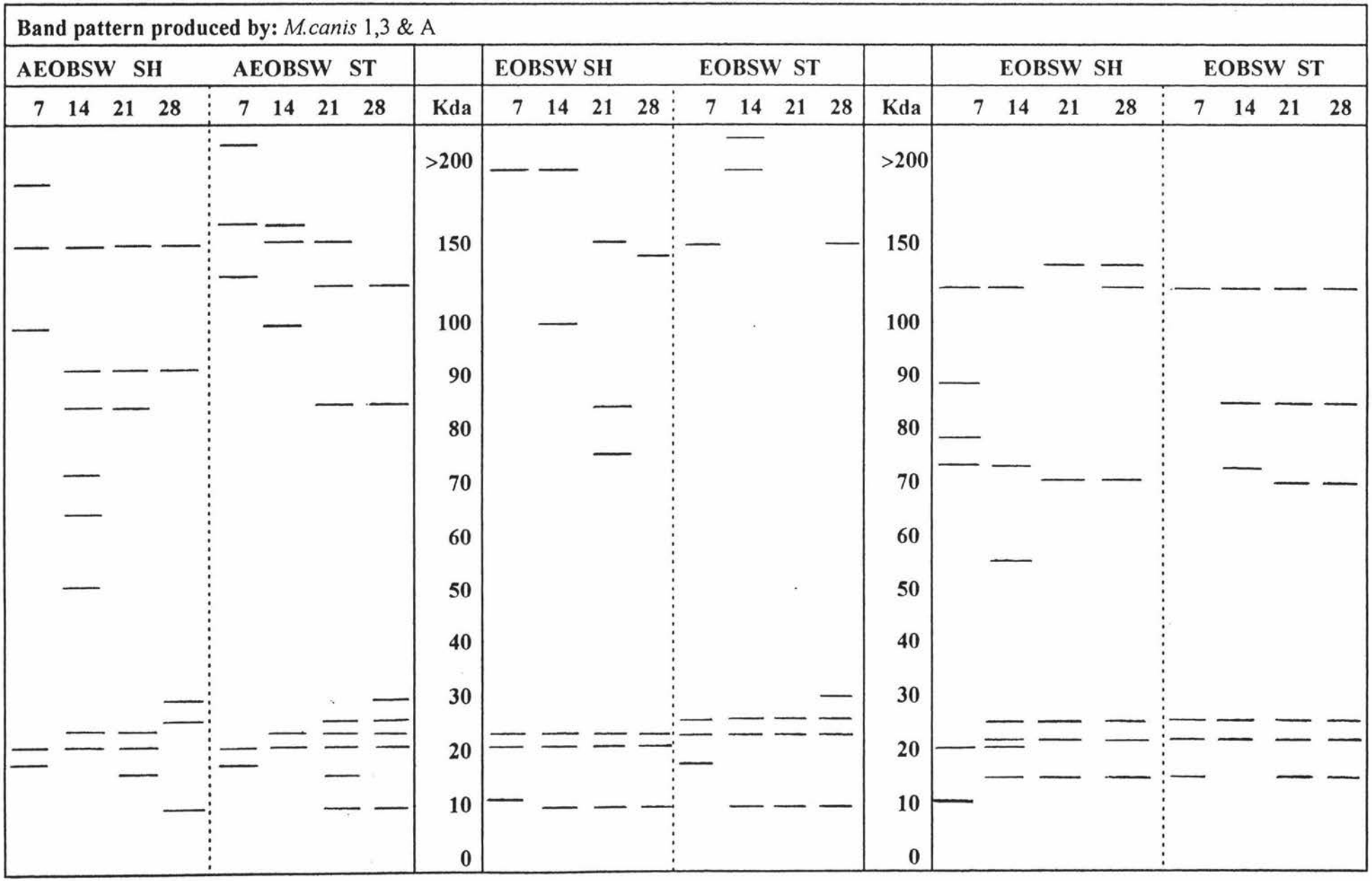
In this medium for both shake and stationary cultures bands were seen in the lower MW range (<50 Kda) making up 63% of total band production.

In shake culture bands at 23 & 21Kda were seen consistently with time through this experimental series. In stationary culture, bands were frequently seen at 151,25,23 & 10 Kda.

EOBSW 97

In this series of gel runs a greater variety of band sizes was seen compared to earlier runs, with 62% of shake culture bands seen in the range between 20 & 100Kda while in stationary culture 68 % of bands were seen in this range.

A number of bands were frequently seen in shake culture at 125,25,23 & 17 Kda while in stationary culture bands were frequently seen at 125, 85,25,23 & 17 Kda with the bands at 125, 25 & 23 Kda being consistently seen over the incubation periods.


Band pattern produced by: M.canis 1,3 & A
AEOBSW SH
AEOBSW ST
EOBSW SH
EOBSW ST
EOBSW SH
EOBSW ST
7 14 21 28
Kda
>200
150
100
90
80
70
60
50
40
30
20
10
0

3.5.2 Gelatinases of *M. cookei* (6)

AEOBSW

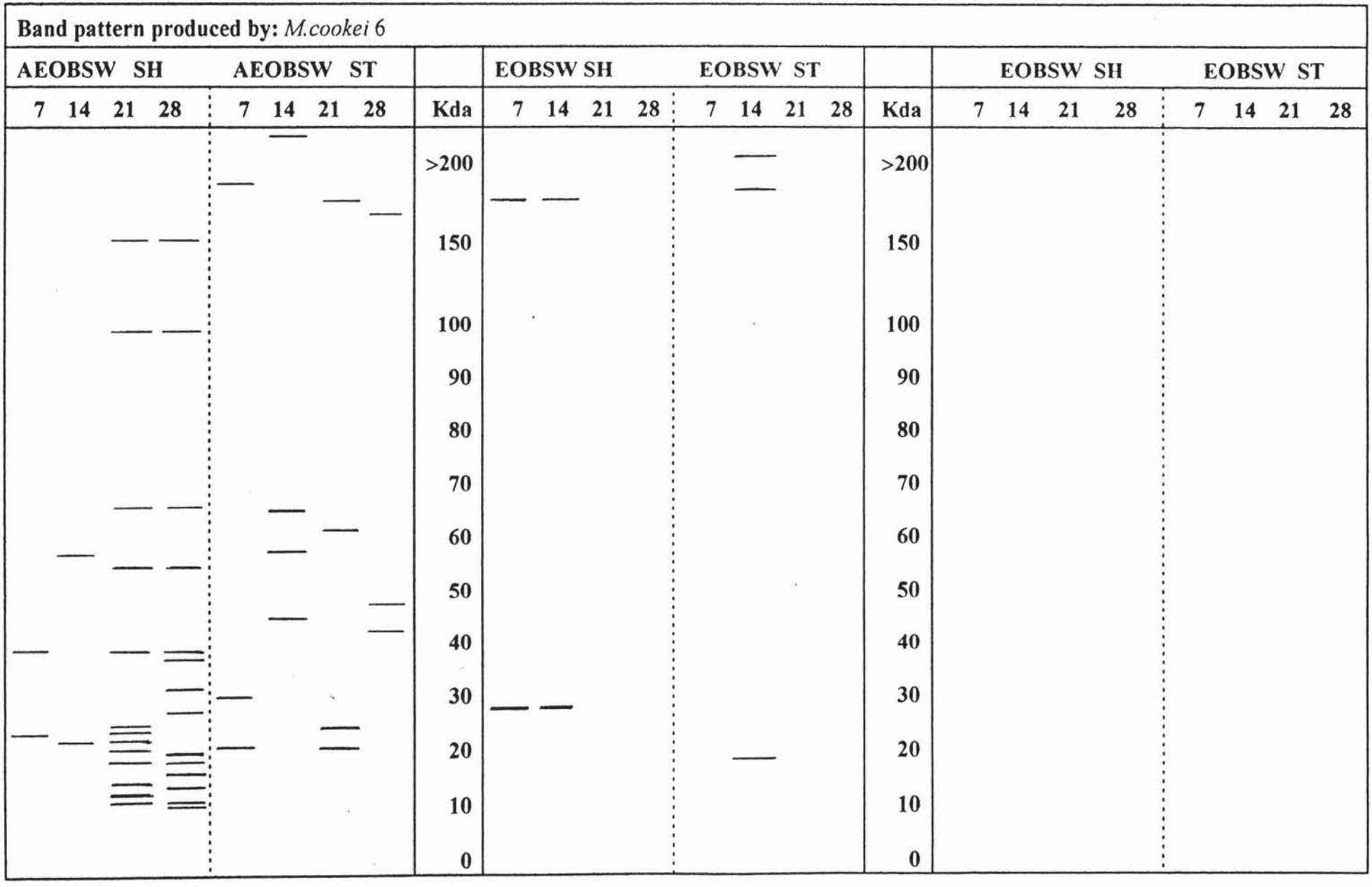
In shake culture mode 75% of bands seen were <50 Kda, with larger bands not appearing until after 14 d incubation. In stationary culture, bands of <50 Kda made up 50% of bands seen with larger bands being seen in all gel runs for this culture mode.

A number of shake culture bands (151, 100, 66, 55, 40, 24, 19 & 12 Kda) were seen on more than one occasion showing some consistency of band of band production for this culture mode. However, in stationary culture only one band at 21 Kda was seen on more than one occasion at 7 and 21 d.

EOBSW

In shake culture, this strain produced only two bands on day7 with these same two bands as well as one other seen at 14 days, following this no bands at all were detected. In stationary culture, three bands were seen at 14 days, with no other bands being detected at all.

Due to poor colony vigour it was decided not to include this strain in further work.


Band pattern produced by: M.cookei 6
AEOBSW SH
AEOBSW ST
EOBSW SH
EOBSW ST
EOBSW SH
EOBSW ST
7 14 21 28
7 14 21 28
Kda
7 14 21 28
7 14 21 28
Kda
7 14 21 28
7 14 21 28
>200
150
100
90
80
70
60
50
40
30
20
10
0

3.5.2a Gelatinases of *M. cookei* (K)

AEOBSW

In shake culture for this medium discreet band production was sparse with bands seen on days 7 & 14 only. One band at 17 Kda was seen on both occasions. In stationary culture, band production was again sparse. Again only one band was seen more than once (200 Kda).

EOBSW 96

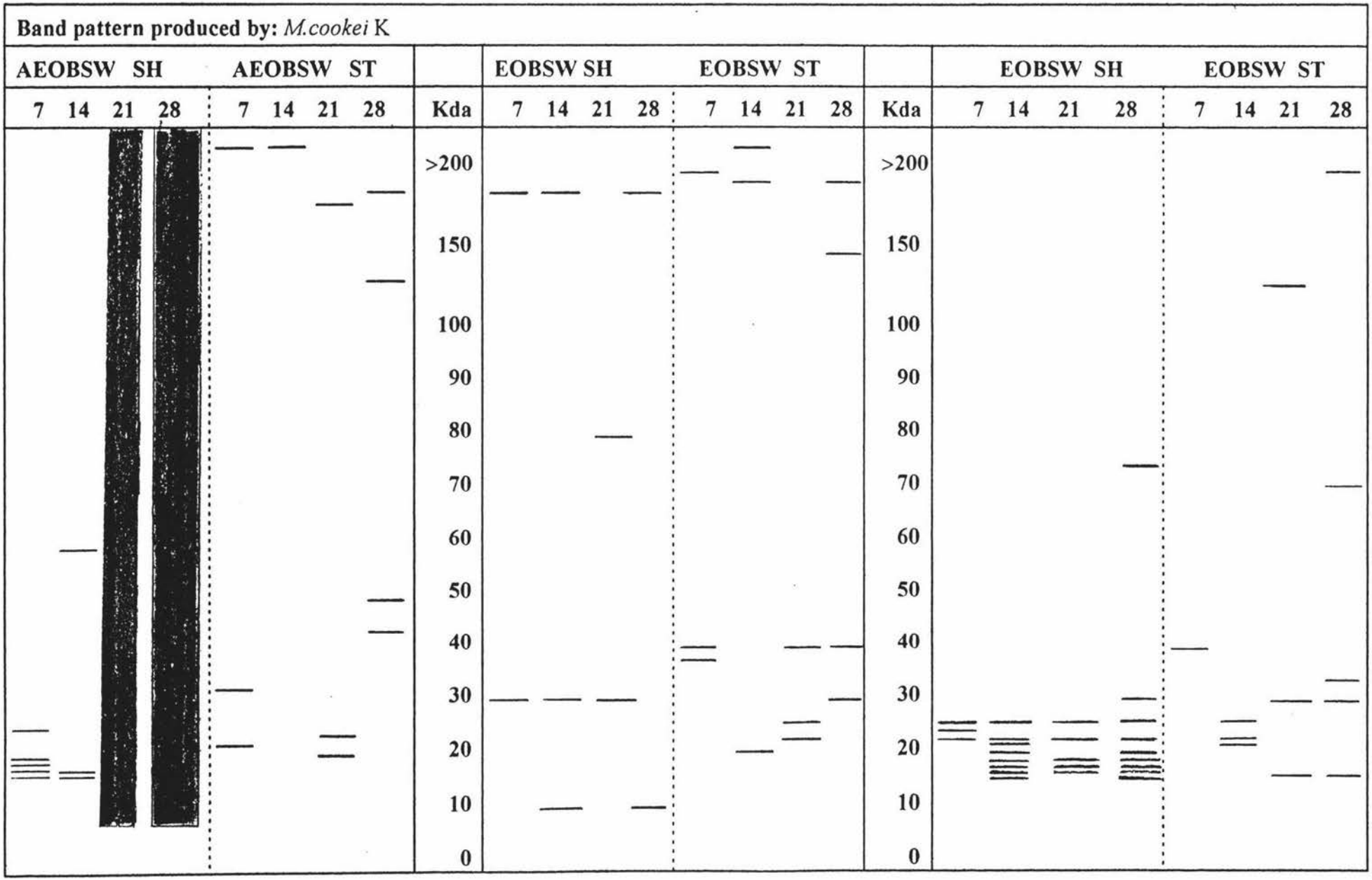
In shake culture for this medium, some consistency of band production was seen with two bands, one at 165 Kda seen on days 7, 14 & 28 and the other band seen at 30 Kda on days 7, 14 & 21.

Band production in stationary culture mode also showed two bands on more than one occasion, one at 170 Kda seen on days 14 & 28 and one at 40 Kda seen on days 7, 21 & 28 Kda.

EOBSW 97

In shake culture with only one exception,one band was seen on day 28 (74 Kda), all other bands seen were <50 kda. Some consistency of production over the culture period was seen with bands at 25 & 23 Kda and bands at 20, 19, 18 & 17 Kda were seen on three consecutive occasions.

In stationary culture, band production was sparse by comparison to the shake culture and only 76% of bands were <50 Kda compared to 96% for shake culture.
In the stationary culture at day 14, bands were seen at 25 & 23 Kda as in shake culture but this was the only occasion on which these two bands were seen.
Other consistencies however, were seen for this culture mode with bands at 30 & 17 Kda seen on days 21 and 28.


Band pattern produced by: M.cookei K
AEOBSW SH
AEOBSW ST
EOBSW SH
EOBSW ST
EOBSW SH
EOBSW ST
7 14 21 28
7 14 21 28
Kda
7 14 21 28
7 14 21 28
Kda
7 14 21 28
7 14 21 28
>200
150
100
90
80
70
60
50
40
30
20
10
0

3.5.3 Gelatinases of *M. gypseum* (B)

AEOBSW

Band production in shake culture mode for this medium was not abundant, with the majority of bands seen (81%) in the smaller Mr. (<50 Kda) range. Some consistencies of band production were seen with bands at 23, 21 & 20 Kda being seen on more than one occasion.

In stationary culture, production of larger Mr bands were noted with bands of 200 & 195 Kda seen at 7 & 14 d. However the smaller bands of < 50 Kda still made up 67% of bands seen for this culture mode. One band was seen at 23 Kda consistently over the culture period while a band at 21 Kda was seen on three occasions on days 7, 14 & 28. A smaller band at 19 Kda was seen on days 7 & 21.

EOBSW 96

In shake culture mode for this medium again few bands were seen and the only band seen on more than one occasion was seen at 28 Kda.

In stationary culture mode band production was more abundant than in shake mode with one band at 23 Kda seen consistently and bands at 100, 30 & 21 Kda seen on more than one occasion through the culture period.

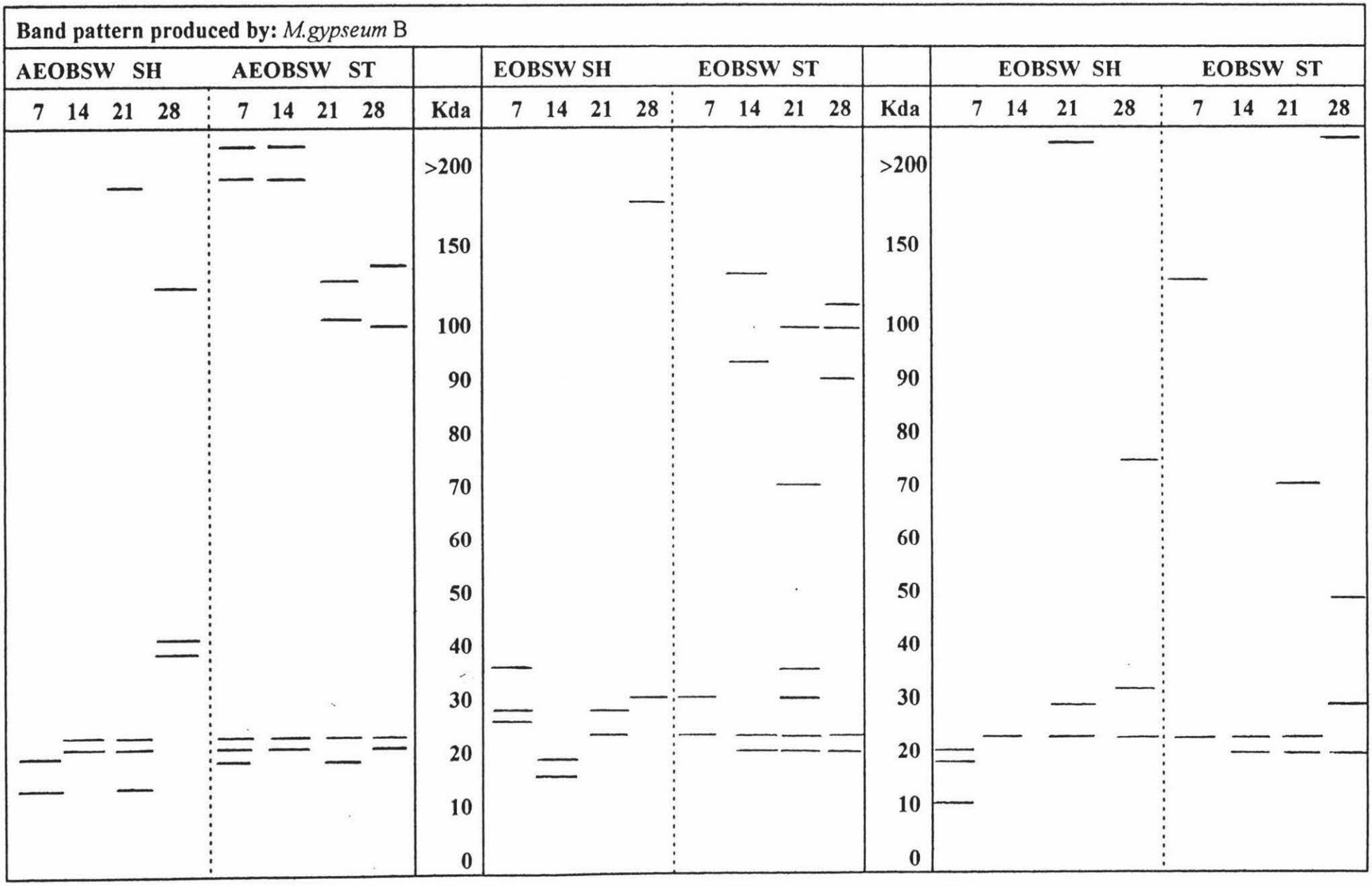
For this series of experiments 73% of bands seen were <50 Kda compared to 41% seen in AEOBSW.

EOBSW 97

In shake culture mode for this series, again few bands were seen with the only band seen on more than one occasion was at 23 Kda.

In stationary culture mode band production was less abundant than was seen in earlier runs. However, again bands were seen on more than one occasion at 23 & 21 Kda.

In this series, the majority of bands (76%) were again <50 Kda, with the most commonly seen bands at 23 & 21 Kda.


Band pattern produced by: M.gypseum B
AEOBSW SH
AEOBSW ST
EOBSW SH
EOBSW ST
EOBSW SH
EOBSW ST
7 14 21 28
Kda
>200
150
100
90
80
70
60
50
40
30
20
10
0

3.5.4 Gelatinases of *M. nanum* (L) (Not illustrated)

AEOBSW

Culture filtrate from this strain generally showed little initial band production over the initial 14 day period, while at 21 days band production was similar for both culture modes and 3 bands in particular were seen frequently in both shake and stationary culture modes (23, 21 & 19 Kda). Bands below 50 Kda made up 83% of the total no of bands produced in this medium.

EOBSW 96

Band production was more apparent in shake cultures in this medium than in the previous series (AEOBSW) with band sizes evenly distributed between 200 - 10 Kda.

In stationary cultures, bands were seen at 14, 21 & 28 days, but at 28 days there was a reduction in band production.

Due to an apparent lack of colony vigour this strain was not investigated further.

3.5.5 Gelatinases of *T. ajelloi* (E)

AEOBSW

Band production in this medium was sparse for both culture modes, with smaller Mr (<50 Kda) bands making up 57% of those seen in shake culture while in stationary culture 100% of the bands were seen at >150 Kda. Consistencies of band production were seen in shake culture for days 7 to 21 (175 & 20 Kda) and in stationary culture for days seven and fourteen (>200 Kda).

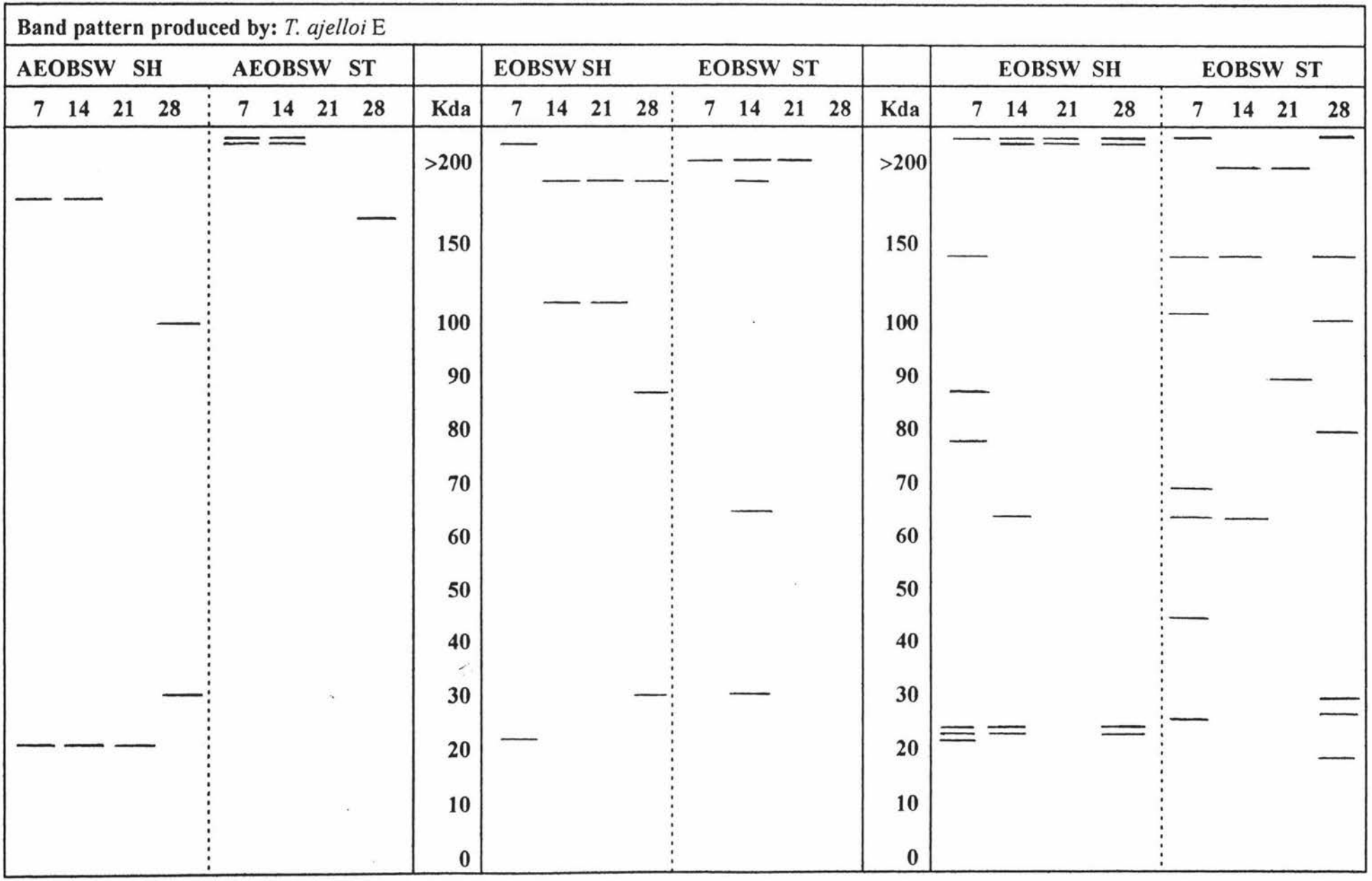
EOBSW 96

In this culture, overall band production increased 20% on that seen in AEOBSW. Consistency of band sizes were seen for some of the larger Mr bands, in shake culture for days 7 to 21 (170 & 112 Kda). In stationary culture a 200 Kda band was seen consistently from day seven to twenty one.

EOBSW 97

In this series, band production was considerably increased compared to earlier runs, with (76%) more bands produced compared to the original AEOBSW series and 2.5 times more bands than was between in the original EOBSW series.

During the 97 series, greater consistency of band production was seen than earlier with there also being some consistency of band sizes between shake and stationary cultures.


Band pattern produced by: T. ajelloi E
AEOBSW SH
AEOBSW ST
EOBSW SH
EOBSW ST
EOBSW SH
EOBSW ST
7 14 21 28
Kda
>200
150
100
90
80
70
60
50
40
30
20
10
0

3.5.6 Gelatinases of *T. mentagrophytes* (D)

AEOBSW

Band production in this medium was good with a number of bands of differing sizes seen for the shake cultures. While most activity was seen at 7 days with five bands being produced, some of these bands were again produced at the end of the incubation period.

In stationary culture, again most activity was seen at 7 days with eight bands being produced. Once again there were bands present on more than one occasion over the incubation period. On day 28 however, extensive smearing was seen in the lane in which this culture filtrate was run and this may have obscured some bands.

EOBSW 96

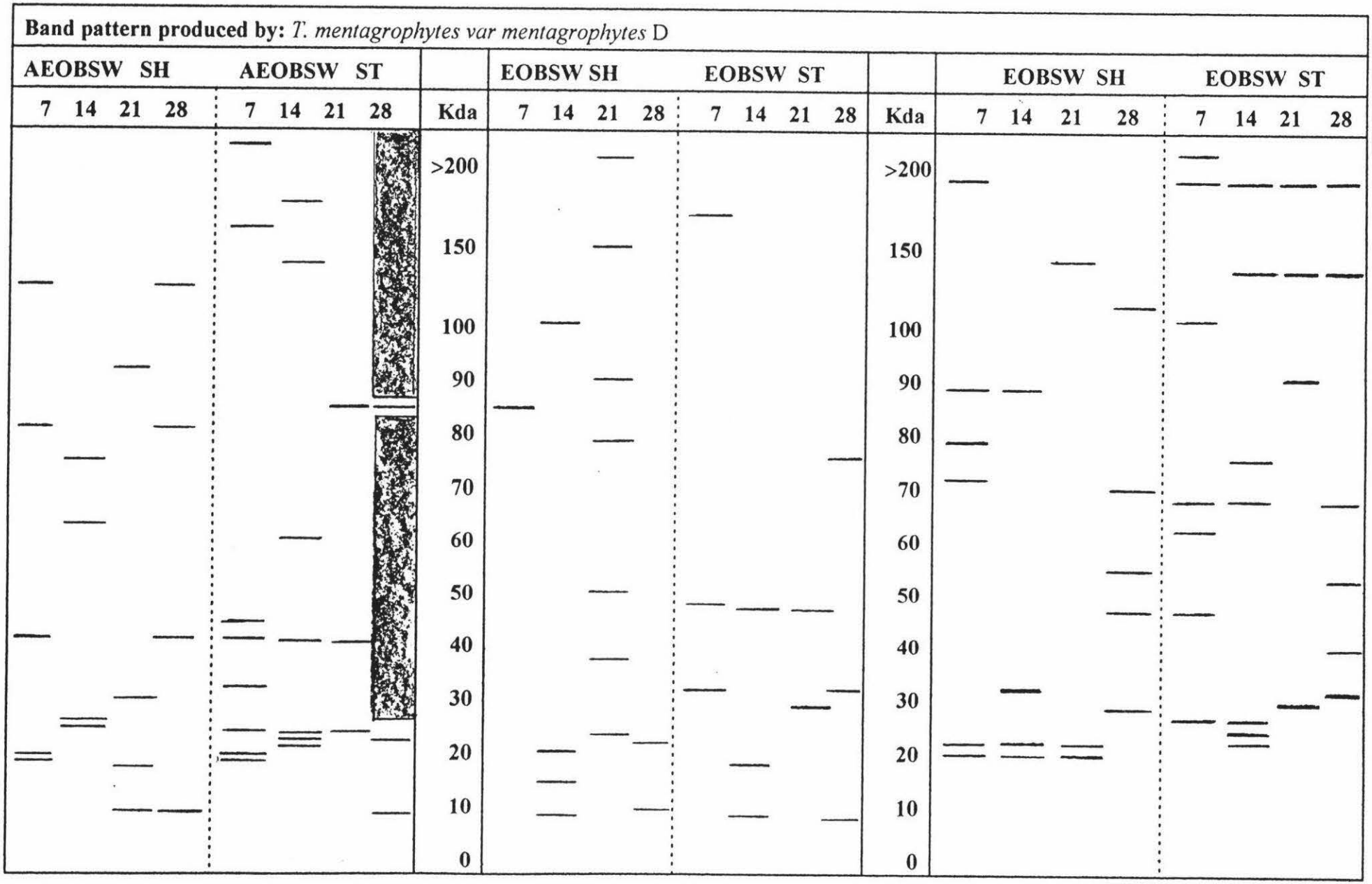
Band production in this medium in shake mode was initially slow with only one band seen, however, activity increased over the next 14 d with seven bands seen on day 21 followed by a a fall in number by day 28.

In stationary culture, band production was not abundant, but some consistency of production was seen.

EOBSW 97

This strain was cultured again 12 months later with little corelation of results when compared to the 1996 run being seen.

However, in the second series more proteolytic activity was seen, particularly in the stationary culture. This culture also demonstrated time consistency of band production particularly among the larger (>50 Kda) bands, with these bands making up 50% of the shake culture bands and 65% of the stationary culture bands respectively.


Band pattern produced by: T. mentagrophytes var mentagrophytes D
AEOBSW SH
AEOBSW ST
EOBSW SH
EOBSW ST
EOBSW SH
EOBSW ST
7 14 21 28
Kda
>200
150
100
90
80
70
60
50
40
30
20
10
0

3.5.7 Gelatinases of *T. erinacei* (M) (Not illustrated)

AEOBSW

The majority of bands produced by this strain in this medium were in the 10 - 25 Kda range with three shake culture bands and four stationary culture bands seen above this and one shake culture band seen at 9 Kda (28 days).

Most frequently produced was a band seen at 23 Kda three times in shake culture and twice in stationary culture.

EOBSW 96

Slightly more band production by this strain in this medium was seen in the shake culture with a greater range of sizes and more bands evident at 14 and 21 d than seen previously. For the stationary culture the same number of bands were seen in this medium as in the AEOBSW stationary cultures.

The smaller bands (>50 Kda) were predominant throughout the trial period and made up 58% of the total number of bands seen. The 23 Kda band seen earlier was seen once in shake culture while in stationary culture there was a band seen twice at 22 Kda.

Overall this strain was not particularly productive and it was decided not to continue with it.

3.5.8 Gelatinases of *T. terrestre* (J) (Not illustrated)

AEOBSW

In both culture modes, the larger sized bands predominated, with only one band seen <50 Kda. A band was seen at 125 Kda in both culture modes and a band at 100 Kda was seen consistently in the shake culture and was seen once in the stationary culture.

In the stationary culture two bands was seen on more than one occasion. However, there was little consistency of band production.

EOBSW

In shake culture for this medium, initially, there were few bands and those that were seen were all >50 Kda, there was little consistency of band production. A band at 200 Kda was seen once in shake culture and once in stationary culture, and a band at 160 Kda seen twice in the shake culture.

Overall, enzyme production did not appear abundant and it was decided to delete this strain from further tests.

3.5.9 Gelatinases of *S. brevicaulis*

AEOBSW

A strong smear was frequently found whenever culture filtrate from this strain was run, and it was thought that this may have obscured some bands within the range of the smear. However, on occasions sharply defined areas of gelatinolysis were viewed within the smear itself, allowing for bands to be measured.

This strain produced a variety of bands particularly in the smaller range, <50 Kda. There was also found to be some consistency of band production with bands at 20, 7 & 6 Kda being seen on more than one occasion for both culture modes.

EOBSW 96

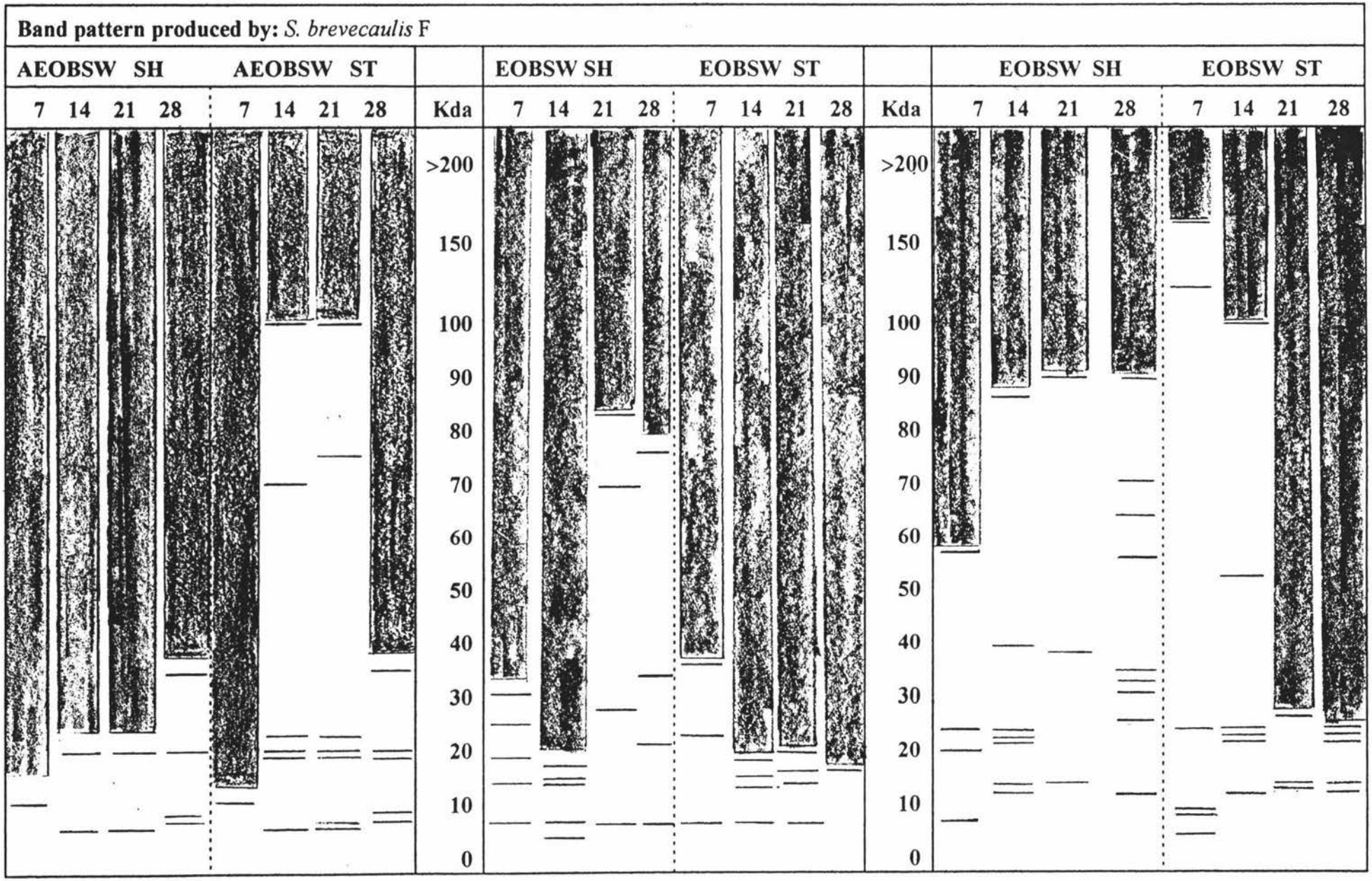
The smear seen in earlier runs was once again evident, but again, some bands were clearly defined within this smear. In shake culture smaller bands were predominant, making up 83% of the bands seen in this culture mode while in stationary culture all bands visualised were <50 Kda. It was noted that a band seen at 7 Kda appeared frequently through this series of gel runs.

EOBSW 97

In this series of gel runs, the smear commonly seen previously was still evident but not as strongly as before, making discernment of larger bands possible.

It was noted that while more bands were seen, fewer of them were in the very small range (<10 Kda) seen previously, although the majority of bands seen (80%) were still <50 Kda.

While a large number of bands were seen for this strain in this series of experiments, there was little consistency of band production seen, with the most frequently seen band being at 23 Kda.


Band pattern produced by: S. brevecaulis F
AEOBSW SH
AEOBSW ST
EOBSW SH
EOBSW ST
EOBSW SH
EOBSW ST
7 14 21 28
Kda
>200
150
100
90
80
70
60
50
40
30
20
10
0

3.5.10 Gelatinases of *A. terreus* (H)

AEOBSW

In both shake and stationary culture modes, most band production was in the lower Mr range (<50Kda), accounting for 90% of the total bands seen in this medium.

Bands at 20, 19 & 18 Kda were consistently seen in both culture modes and, the 23 Kda band seen commonly in other cultures was also seen twice in stationary culture at 14 and 21 days.

EOBSW 96

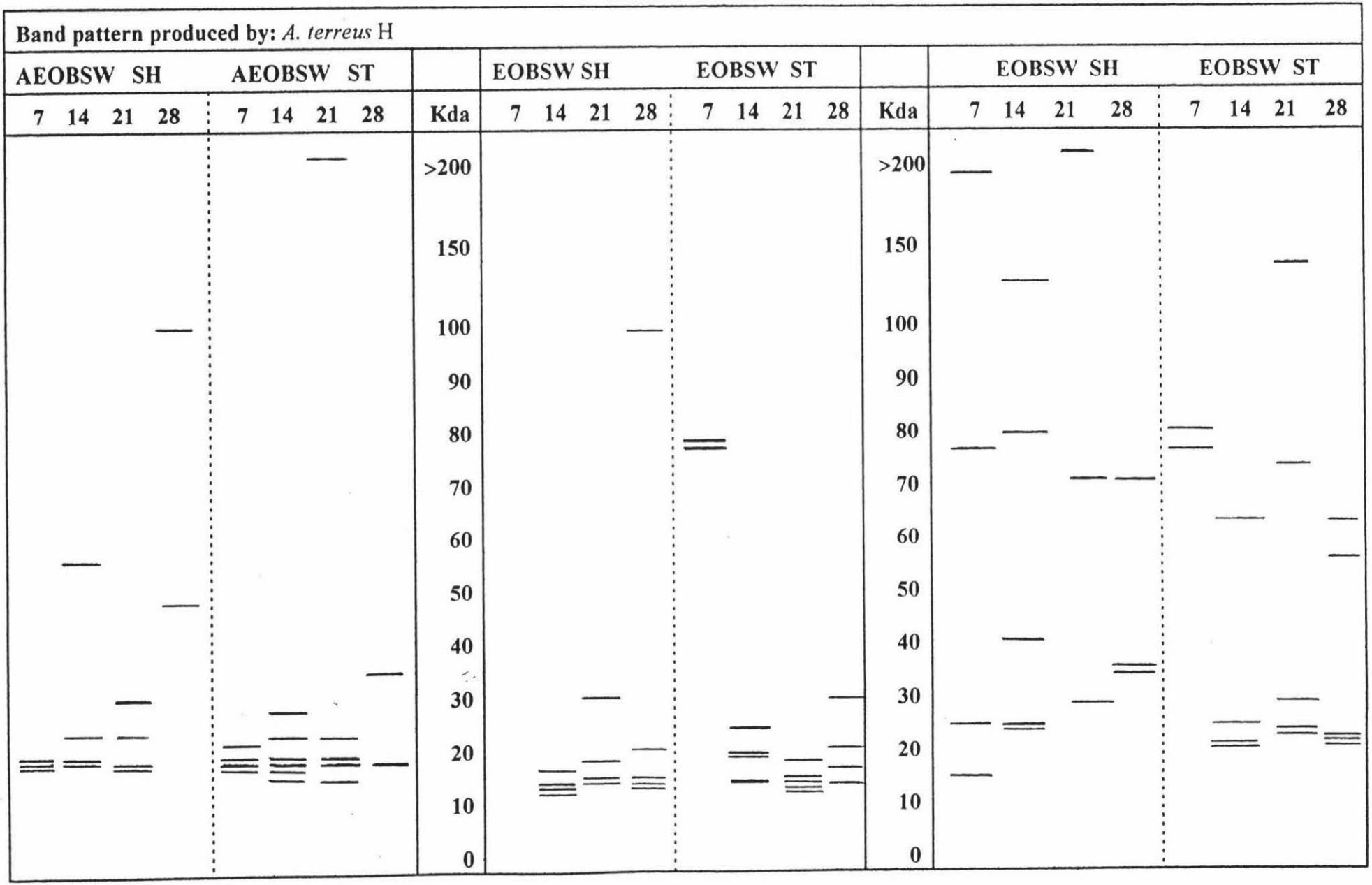
In this medium for both shake and stationary culture modes, once again the majority of band production was in the lower Mr range (<50 Kda) with these making up 89.3% of the total bands seen in this series of gel runs.

Bands were seen at 19 & 20 Kda in this medium, but not with the same consistency as seen previously for the AEOBSW runs.

EOBSW 97

Total band production in this series was increased by three bands compared to the previous run (96) and differences were noted in the range of the bands produced. Only 51.6% of bands were <50 Kda compared to 89.3% for the 96 series.

There was little consistency of band production with one band (25 Kda) seen twice in shake culture at 7 and 14 d and a 25 Kda band was seen twice in stationary culture at 14 and 21 d, a 23 Kda band was seen at 21 and 28 days.


Band pattern produced by: A. terreus H
AEOBSW SH
AEOBSW ST
EOBSW SH
EOBSW ST
EOBSW SH
EOBSW ST
7 14 21 28
7 14 21 28
Kda
7 14 21 28
7 14 21 28
Kda
7 14 21 28
7 14 21 28
>200
150
100
90
80
70
60
50
40
30
20
10
0

3.5.11 Gelatinases of *C. keratinophylum* (I) (not illustrated)

AEOBSW

Band production in this medium was mainly of larger Mr. (>150 Kda) bands with only one smaller band (31 Kda) seen in shake culture at 28 d.

There was some consistency of band production seen with the bands of 200 & 165 Kda seen twice in shake culture while the 200 Kda band was seen twice in stationary culture and the 165 Kda band once.

EOBSW

Band production in this medium was sparse with only three bands seen in shake culture over the incubation period and five bands seen in stationary culture.

There was no apparent consistency of band production demonstrated by this strain in this medium.

3.5.12 Gelatinases of *D. chlamydosporium* (7) (not illustrated).

AEOBSW

In shake culture for this medium bands were seen in a range of sizes with only one band (18 Kda) being seen on more than one occasion.

In stationary culture a particularly large range of bands were seen over the incubation period with 38 % being between 50 - 100 Kda, 21% between 20 - 50 Kda and 38 % <20 Kda.

In the stationary culture a number of bands were seen on more than one occasion (84, 75, 23, 18 & 17 Kda).

EOBSW

In this medium band production was sparse by comparison to that seen in theother culture medium; in shake culture in particular very few bands were produced.

In stationary culture, fewer bands were seen than in the AEOBSW series. The smaller bands (<20 Kda) made up 60% of bands observed in shake culture in the EOBSW medium.

There were some consistencies of band production seen over the incubation period with a number of smaller bands seen on more than one occasion (190, 16,13, 12 & 11 Kda).

This strain became difficult to subculture and it was decided to delete it from further experiments.

3.5.13 Gelatinases of *D. chlamydosporium* (8)

AEOBSW

This strain produced a number of bands in both shake and stationary cultures particularly those of smaller Mr. Bands <50 Kda made 65% of bands seen in shake culture and 61% of bands seen in stationary culture.

Little consistency of band production was seen in shake culture although a band was seen at 19 Kda on both day 7 and 14 and a 14 Kda band was seen on day 7 and 21 in shake culture.

In stationary culture, the largest range of bands was seen and bands at 20, 18, 16 & 14 Kda were seen on more than one occasion.

EOBSW 96

Less bands were seen in this medium than previously with the bands of smaller Mr. (<50 Kda) again being predominant. They made up 68% of bands seen in shake culture and 81% of bands seen in stationary culture.

Some consistency of band production was seen in the shake culture with a band at 150 Kda seen on days 7, 14 and 28. In stationary culture, bands were seen at 13 & 12 Kda on days 21 and 28.

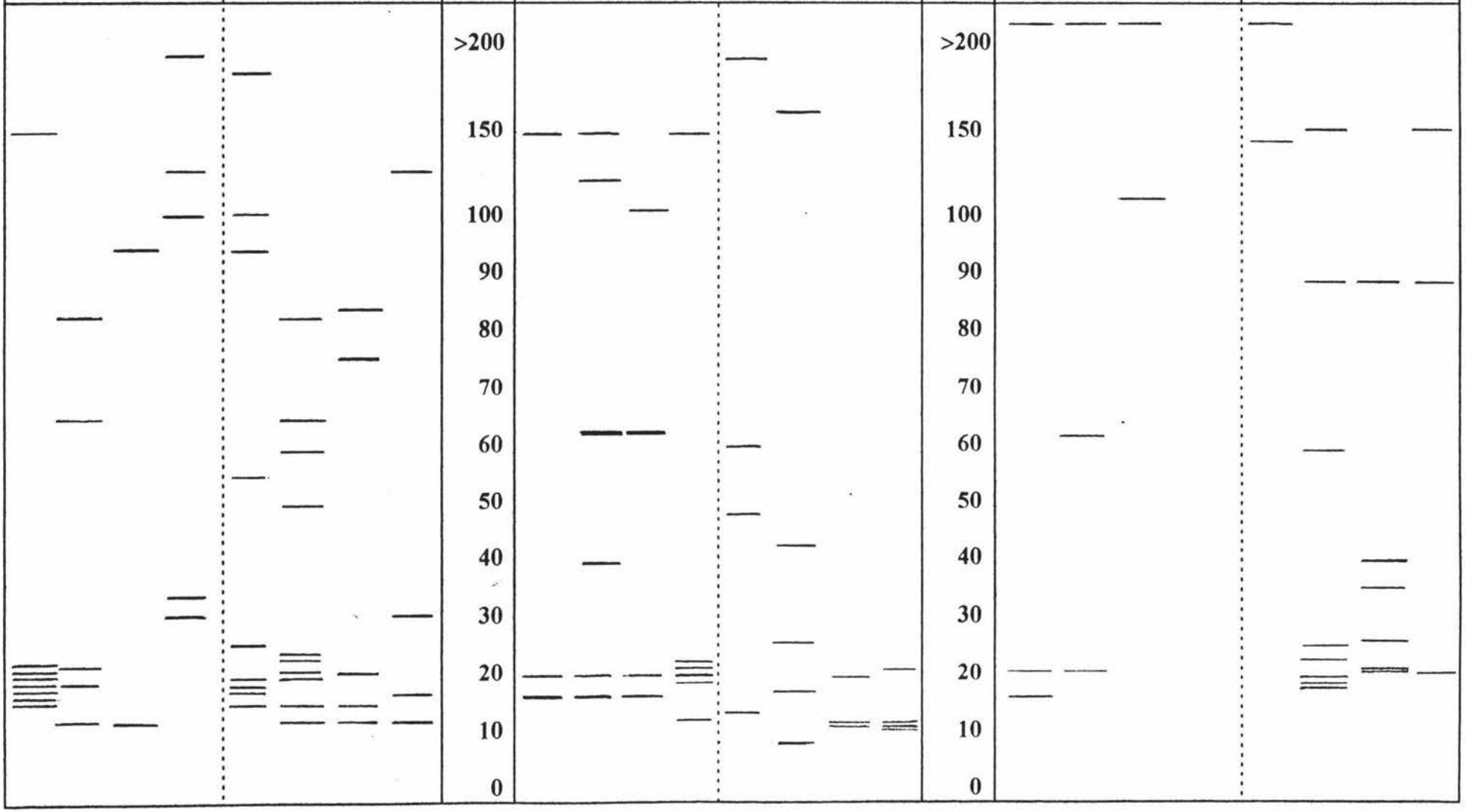
EOBSW 97

There was less production for the shake mode of culture in this series with 50% less bands seen. In the stationary culture mode however, there was a slight increase in the number of bands seen and the range of band sizes. More bands between 20 - 50 Kda were seen.

There was some consistency of band production seen in shake culture with a >200 Kda seen on days 7, 14 and 21 and on days 7 and 14 a band was also seen at 21 Kda.

In stationary culture two bands were seen (90 & 20 Kda) on days 14, 21 and 28.

Band pattern produced by: *Diheterospora sp.* 8



CHAPTER 4
DISCUSSION AND CONCLUSIONS

4.1 The Soil as a Natural Reservoir of Pathogenic Fungi

The examination of the soil for keratinolytic fungi involved keratin baiting soil samples obtained from the Massey University campus grounds and outlying areas of the Manawatu and one sample from Taupo.

Much of this area had been previously researched for keratinophilic fungi by investigators in this area. As well as acquiring fresh strains for examination, this study also contrasted the results of previous workers concerning the local distribution of species.

It is thought that the distribution of keratinophilic fungi may be associated with the type of keratin material present in the soil (De Vroey, 1968), or that 'animalisation' (i.e. the enrichment of soil with keratin or other organic matter) of the environment may create conditions suitable for the growth of keratinophilic fungi. (Batelli *et al.*, 1978, Mercatini *et al.*, 1978,)

While this may be true of pathogenic fungi, it has been determined that the type of keratin is not critical in determining the distribution of saprophytic keratinophilic species found in soils, as many of these fungi have a biological role as agents of decomposition (Male, 1990). They are able to utilise a number of organic substrates, with keratin utilisation only a useful character when it is necessary to compete for nutrients with other soil micro-organisms present.

It was observed that "keratin-in-contact with soil" (Griffin, 1960b) produces a characteristic fungal flora, with the initial visible flora being highly competitive saprophytic fungi with the ability to use less complex, more readily attainable nutrient substrates. Frequently encountered here were *Cladosporium,Penicillium & Fusarium spp.*.

Within ~ 25 days the majority of these species had disappeared and the keratinophilic species became evident with *M. gypseum & T. ajelloi* being commonly isolated. Together they made up 61.5% of the keratinophilic species isolated from the soil in this study.

Of the areas sampled, 46.7% was farmland or land involved in the support of a variety of farm and domestic animals, and, with the exceptions of the Moutua milkshed and the sample from the Taupo showgrounds, all of these soils yielded keratinophilic fungi and a large number of non-relevant fungi also.

The Moutua milkshed sample may well have been carrying a particularly high organic load, as the sample was taken from the cattle race leading to the milking shed, which may account for there being no growth of fungi of any species. The sample from Taupo was taken from a popular animal show venue and during an event. The reason for the failure of any type of fungi to grow from this soil is unknown, but may be related to the large amounts of pesticides and skin treatments being utilised by various exhibitors.

The areas surrounding the University proved to be a good source of keratinophilic fungi, probably because there is a great deal of farmland associated with the campus and the considerable movement of students to and from the associated farmland must enhance the spread of both propagules and keratinous debris. Overall, from 30 soil samples, 24 were positive for keratinophilic fungi.

Notable was the frequent isolation of *M. gypseum* from the soils sampled, with 12 positive isolations of *M. gypseum* from 24 samples in which growth had occurred. Seven strains were isolated singly and another five strains were isolated in conjunction with *T. ajelloi.*

When investigated by Marples (1965), New Zealand soils, particularly South Island soils were found to have a very low incidence of *M. gypseum,* and when local sites were investigated by Lee (1979), this species was isolated only from 13 of 153 sites (13% of keratinophilic species found in that study). In 1994, when investigations into the distribution of keratinophilic species was carried out by Simpanya, only 5 positive isolations were made from 236 samples, this being an isolation rate of only 1.7% (Simpanya 1994).

The absence of zoophilic and anthropophilic dermatophytes in the soils examined is probably due to their inherent inability to adapt and hence survive in the soil. It has been found that anthropophilic and zoophilic species are lysed by the action of micro-organisms in soils (Grin and Ozegovic 1963), while geophilic species show more resistance to such antifungal activities.

This antagonism from other microbes is probably a factor in decreasing the survival capabilities of the more pathogenic species in the soil, thereby reducing the chances of isolation.

Among the other less frequently isolated keratinophilic species which can be obtained from hair baited soil and which were used in these studies are *Aphanoascus terreus, Scopulariopsis brevicaulis & Diheterospora spp.*.

The *A. terreus* species isolated in this work from samples J1 & J2 Himitangi beach (Results table 3.1) was originally identified from microscopic examination of 14 day old hyphae and spore morphologies as a member of *Chrysosporium.* However, at 21 days cliestothecium development was noted and further subculturing strongly suggested that it was a member of *Aphanoascus.* Further microscopic examination of ascospores and comparisons with the published photographic descriptions of Cano & Guarro (1990) helped to confirm that the species isolated was indeed *A. terreus.*

A number of identified species of *Aphanoascus* have been named as the teleomorphic states of various keratinophilic *Chrysosporium spp.*. These species are commonly isolated from soil and are occasionally pathogenic to man (Rippon *et al.*,1970) and other animals

Scopulariopsis sp. are able to attack a wide range of organic substrates including keratins. It is also able to decompose arsenic compounds, allowing it to grow on paint or certain types of wallpaper containing arsenic (Gravesen 1994). *S. brevicaulis* is one of the non-dermatophytic species often found in human infection (Velez, 1997). It is occasionally found in skin infections but is more common in onychomycosis or infection of the nails, in particular, the toe nails. This species has also been found in eye and pulmonary infections of immunocompromised patients.(Balows *et al.*, 1991).

The genus *Diheterospora* is commonly found associated with keratin baits of soil samples from the Manawatu (Simpanya, 1994). It was erected by Kamyschko (1962) on two species, *D. heterospora & D. catenulata.* Barron & Onions (1966) later showed this genus to be conspecific with *Verticillium chlamydosporium* described by Goddard (1913) and the type species to date is now regarded as *D. chlamydosporia* (Barron & Onions 1966).

This species has a *Verticillium*-like phialidic anamorph and also produces large, muriform, terminal aleuriospores which are golden brown in mass and give colonies a creamy colour. Kamyschko also described *D. catenulata*, which is very similar to *D. chlamydosporia* in general characteristics but differs in producing a *Paecilomyces*-like phialidic anamorph (Barron, 1984).

Members of this genus are known to infect snail eggs (Barron & Onions, 1966). They have also been found to have an ovicidal effect of the eggs of *Ascaris lumbricoides* (Lýsek, 1978), and are very common parasites of bdelloid rotifers (Barron, 1973, 1980, 1984). Scanning electron microscope observations have confirmed that the first phase of ovicidal action involves the formation of perforation organs, on the surface of the eggs, following invasion by fungal hyphae, enzymatic activity occurs damaging the shell structure (Lýsek ,1978), analogous perhaps to dermatophyte invasion of keratin structures.

4.2 Identification/Morphological Studies.

These were carried out for initial identifications via traditional methods and to observe any possible arthroconidial growth induced in the known pathogenic species or pseudo-parasitic growth in the non-pathogenic species under artificial conditions.

All cultures selected and cultured on SDA at 25° C were found to have both gross and microscopic morphology which conformed to well to published features.

Liquid cultures were also examined. In SDB shake cultures, a mycelial mat did not form as such, instead, there was pellet formation of hyphal growth only seen submerged in the medium. These pellets enlarged rapidly. Where there was exposure to air, basic gross colony characteristics were seen. These pellets were generally pigmented similarly to that seen on solid media, with the exception of *S. brevicaulis*, which produced a white waxy pellet rather than a brown or grey pellet as may have been expected.

exception of *M. gypseum,* see below) found to be hyphal, with no visible spore development, either saprophytic or parasitic and this same held true also for the *Trichophyton spp.*. However, in the case of *T. mentagrophytes var mentagrophytes,* a number of 'nodules' were observed extending horizontally from the lateral surface of hyphae. (Plate 3.2.5d).

A similar lack of conidia or arthrospores occurred in BSW shake cultures. In contrast for the non-dermatophyte strains examined, microscopic examination showed both hyphae and spore production as seen on the solid medium, the saprophytic morphology.

In the case of *M. gypseum, m*icroscopy studies revealled abundant atypical growth on more than one occasion large arthrospore/chlamydospore-like structures were formed (Plates 3.2.3g, 3.2.3e). Comparisons of the morphology seen with that published by Weigl *et al.*, 1979, suggested that this may be the morphology often referred to as "pseudo-parasitic".

Thus these studies in most cases did not confirm the findings of previous researchers who utilised shake culture to induce the arthrospore growth morphology of a pseudo-parasitic stage (Raubitschek, 1955, Evron-Maoz *et al.*, 1960).

However, other researchers have had some success in inducing such a morphology in dermatophytes and opportunists by manipulations of the CO_2 concentrations of the culture incubation atmosphere (Zurita *et al.*, 1987,Weigel *et al.*,1979, Rashid *et al.*, 1995). The work done by these researchers indicate it may be possible to actively control the morphology of a fungal culture using this parameter. Such studies, if successful, in these areas will aid the development of an *in vitro* model suitable for the study of superficial fungal invasion.

4.3 Indicators of Growth and/or Activity

The initial investigations and microscopic examinations of cultures and spore suspensions revealed that spores were sparse and the morphology often atypical. It was thought that the long term use of antibiotics may have been responsible for this.

The antibiotic chloramphenicol and the selective antifungal agent cycloheximide, frequently used in selecting for dermatophyte fungi had been added to culture media in an attempt of overcome problems of contamination.

It was decided to culture the strains in the absence of antibiotics. The results presented demonstrate that the number of spores produced markedly increased following subculture on LMA or dilSDA slopes. The species involved were found to have recovered their original morphology and further studies indicated that proteolytic enzyme production had also recovered (Results 3.4).

As enzyme production seemed related to the production of spores (perhaps a reflection of adequate fungal growth), the practice of spore counting was adopted to ensure sufficient spores were always present in subsequent inocula used for enzyme production cultures.

Although spore concentrations varied from species to species, numbers were always high and concentration was not adjusted to a standard level, as in theory, infection may arise from adherence and germination of a single spore or growing hyphae.

Recent research by Sobel *et al.*, (1998) has shown that in order for a minimum foot infection dose rate of 50% ($MFID_{50}$) of Hartley strain guinea pigs to be achieved, either only 280 arthroconidia or 315 microconidia were required to induce infection on every foot consistently and synchronously.

Also, observations of medium pH in these studies served as an indicator of growth and secreted enzyme activity. It has been previously noted by other researchers that the breakdown of keratin into proteins, peptides and amino acids results in changes to the pH of the medium (Deshmukh *et al.*, 1981, Tanaka *et al.*, 1992). Thus, changes in medium pH would indicate that some kind of activity was occurring within the culture vessel.

Generally, in LMB shake cultures, there was an initial increase in pH of all cultures with the exception of the *D. chlamydosporium* which showed an initial decrease (followed by an increase at 14 days). *M. cookei & A terreus* showed a sudden lowering of pH at 14 days whereas the values for the other cultures continued to increase.

In contrast, in stationary mode, all cultures showed an initial decrease in pH over the first 7 days and, with one exception, an increase by day 14. The exception was S. *brevicaulis* in which the medium pH continued to decrease to day 14.

The BSW shake cultures all showed a steady increase in pH over the initial 7 - 14 day period, indicating good growth and activity with further increases or stability up to day 28.

The BSW stationary cultures however, all showed an initial decrease in medium pH followed by an increase then a levelling off over the remainder of the experimental period, perhaps indicating that autolysis was occurring.

Proteinases expressed after seven days growth when the pH falls may be the more acidic proteinases and those expressed from around day 14 onwards when the pH rises may be more neutral to alkaline enzymes. Tanaka *et al.*, (1992) reported that in a keratin-supplemented medium *T. rubrum* showed differences in activities of acidic and neutral proteinases as the age of the culture increased, with acidic proteinases being highest during the first two weeks of the culture and a neutral to alkaline proteinases increasing after the third week when culture pH increased from 6 to 7.

Thus, overall, pH was a good indicator of activity occurring within a culture.

4.4 Proteolytic enzyme production

Initially, a gelatin-hydrolysis plate method was trialed in this study as a general indicator of proteinase expression by various isolates. It was not found to be of much value in relation to the concept of high proteolytic enzyme production by dermatophytes. The proteolytic activity for some species of saprophytes and opportunists was much higher than for many of the pathogenic species, therefore, this technique merely indicates that these saprophytes could be better able to utilise general proteinaceous substrates *in vitro.* However, the second method to indicate proteolytic (gelatinolytic) activity, charcoal-gelatin discs, demonstrated greater efficiency and effectiveness. The visible disintegration of the discs quickly revealed the presence or otherwise of proteolytic enzymes in the culture liquid.

Studies of the expression of proteolytic enzymes produced by the different keratinophilic species examined were in part aimed at determining which, if any, proteolytic bands or groups of bands could be found common to all. It was considered possible that similarities in proteolytic enzyme production between the various strains and species may reflect the ability to utilise keratins and also possible virulence determinants of importance in determining the pathogenic abilities of some species.

One factor which appeared to have a strong effect on the expression of proteolytic enzymes was substrate. SDB medium contains peptone and dextrose which are rich sources of peptides and sugars for a fungus to grown on. However, during the course of early studies, the addition of cycloheximide and chloramphenicol to media to control contamination, (over a 12 month period) reduced colony vigour as reflected by atypical colony morphologies and reduced proteolytic enzyme production.

The use of nutritionally rich media for the re-establishment of typical gross and microscopic colony morphology is well established. In this instance various lactrimel media, were used not only for this purpose but also to examine the suitability of this medium for the re-establishment of proteolytic enzyme production when this ability had appeared considerably reduced.

With few exceptions, an increase in proteolytic enzyme production detectable by SDS-PAGE was seen for strains cultured in LMB compared to that seen using SDBXX with a total overall increase in stationary cultures of ~ 80% observed.

Among the *Microsporum spp.*, examined , the number of bands produced by each strain was increased 88% (ST cultures only) in lactimel broth when compared to band production in SDBXX. Also in many cases, gelatinolytic activity was stronger with bands being more clearly observed. For this genus, the number of bands produced in either the shake or stationary LMB cultures, did not appear significantly different, although MW of bands varied week to week (Results 3.4).

The *Trichophyton spp.*.examined showed mixed reactions to the various culture modes with *T. mentagrophytes* initially produced four bands in SDBXX while in LMB cultures, up to eight were seen in stationary culture at 14 days. The type of medium used did not appear to have a significant effect on the number of bands produced by the *T. mentagrophytes* strain examined. This species appeared able to produce well resolved bands regardless of the culture medium.

T. mentagrophytes strains have undergone extensive examination by various researchers and this group of fungi have been found to be extremely adaptable with the ability to colonise a large number of substrates. (Perera *et al.*,1997, Rashid *et al.*, 1995, Samdani *et al.*,1995, Deshmukh *et al.*, 1981, Yu *et al* 1969).

The *T. ajelloi* strain investigated showed little increase in the number of bands produced overall in LMB in ST. culture compared to those produced in SDBXX, but, extra bands were produced in the SH. culture. In contrast, *T. terrestre and T. erinacei* both showed substantial increases in the number of bands produced in LMB compared to results found using SDBXX cultures, with both culture modes providing clearly resolved bands.

Among those species cultured on SDB without antibiotics prior to culture in LMB, it was noted that bands found in *M. canis* were similar in size to three of those found by Simpanya (1994) who detected six bands in SDB SH. (122, 64, 62, 44, 36 and 28 Kda) and to those found by Palmer (1995) who also detected six bands (85, 61, 40, 36, 27 and 16).

In those instances where strains were cultured in SDB SH mode without antibiotics prior to culture in LMB, increases in the number of bands seen were not dramatic. With *M. canis* an increase of only 20% was seen while *M. cookei* showed no change in the number of bands observed and the *D. chlamydosporium* strains showed <10% increase in amount of bands seen overall for the 14 day period. It was noted that many of the smaller bands seen were of similiar sizes (20 - 30 Kda) in all 4 species.

Similar results were obtained with *A. terreus, C. keratinophilum* stains examined. The *S. brevicaulis* strain examined produced a large number of bands and frequently, extensive areas of gelatinolysis in the upper portion of the gels were seen.

Some investigators in these areas have found a range of proteolytic enzymes have been produced in response to manipulations of nutrient concentrations e.g. Apodaca *et al.*, (1989a,1989b)found that they could manipulate the sizes of the proteolytic bands produced by *T. rubrum* in stationary cultures, by manipulations of the sulphur, carbon, phosphorus and nitrogen content of the culture medium.

BSW medium has been found by previous researchers in this laboratory to be an effective medium to detect gelatinases and has previously allowed consistent demonstration of the component bands. Researchers in other laboratories have also detected proteolytic enzyme production utilising various other keratin sources e.g. Tsuboi (1994) used a liquid medium supplemented with human hair and found *M. canis* expressed three keratinases (MW 48, 34 and 32.5 Kda), while more recently Mignon *et al.*,(1998) using cat-hair containing basal medium isolated a 31.5 Kda protease secreted by an *M.canis* strain isolated from an infected cat.

When selecting a substrate, it was decided that sheep wool was the most suitable for these studies due to the lack of medulla and other easily digested components of the fibres.

One of the aims of the current studies was to observe if possible reproduciblity of results when comparing the current work with that done previously by Simpanya (1994) and Palmer (1995). The *M. canis* control strains used had also been utilised by these researchers.

Mixed results were seen. Consistent with previous findings, all strains produced at least 6 different bands. All strains were able to consistently produce the same sized bands in the present experiments. While band sizes appeared to differ from those found by previous researchers, some similarities were noted e.g. Palmer, using BSW medium detected in *M. canis* cultures, bands at 64.5, 51.5, 34, 30,15 and 13 Kda, while in this series similar sized bands were found between 74 and 65 Kda as well as bands found at 10 and 17 Kda. This may well be an artifact of the technique itself, or due to experimental error.

Variations was also found from year to year e.g. using *M. canis*, the 1996 series regularly revealed bands of MW >150 Kda in both culture media, while in the 1997 series, these bands were not detected at all, also for the *A. terreus* strain, during the 1996 series, only one band was seen >101 Kda, while in 1997, 4 bands >100 Kda were seen.

In considering the genus *Microsporum.* it was noted that bands at 23 & 21 Kda most commonly occurred among *M. canis, M gypseum & M. nanum.* These bands appeared at some stage in all BSW media examined and in both culture modes. It was also noted that the above three species produced other bands of somewhat similar sizes, particularly in the 10 - 20 Kda range.

These 23 & 21 Kda bands were not commonly seen in the *M. cookei* cultures, although they were seen in strain 'K' during the 97 gel runs. This could be a reflection of the fact that this particular strain was isolated from an animal source as opposed to a soil source.

Among the *Trichophyton spp.*were again noted, except in *T. terrestre* bands at 23 and 21 Kda. *T. mentagrophytes* and *T. erinacei* produced a variety of bands with approximately equal numbers of bands found both above and below 100 Kda. Both species are known to be common pathogens of animals and have occasionally been found in human infection (Baxter *et al.*, 1980). *T. ajelloi* and *T. terrestre* were seen to produce a larger number of high MW (100-200 Kda) bands quite consistently. Investigations by Apodaca *et al.*, (1989a, 1989b) of *T. rubrum* have suggested that the larger MW bands are more strongly keratinolytic. Given that these species are commonly regarded as geophilic saprophytes and are only rarely found in human infection, it is possible that this is a reflection of the environmental niche these species commonly fill as decomposers of keratinous debri in nature.

Among the other species examined, overall band sizes were variable. Showing bands predominantly in the smaller MW range, including 23 and 21 Kda. The *C. keratinophylum* strain, however, produced bands almost exclusively in the larger MW range with only three bands <50 Kda seen across the entire experimental series. While strong consistencies were seen in the larger bands with the 200, 180 & 165 Kda bands being seen for the duration of the initial experimental series, colony vigour was poor and subculture difficult and this strain was not selected for further investigation.

The *S. brevicaulis* strain very actively produced proteolytic (gelatinolytic) enzymes and large amounts of smearing was consistently seen in the upper portion of any lane running this culture filtrate. The most readily discerned bands were seen <50 kda, with this strain also producing bands at 23 & 21 Kda on occasion.

The *Diheterospora spp.* produced a wide range of band sizes including bands seen at 23 and 21 Kda, with some consistency of band production seen between strains. Unfortunately, strain 7 had displayed poor colony vigour over the experimental series and was unable to be subcultured for further examination during the second series of gel runs.

Apodaca *et al.*, (1989a, 1989b) have also reported finding various sizes of proteases as well as large smears and have suggested that many of the smaller bands may represent proteolytic cleavages of larger or other proteinases. High MW smears could also represent multimers of lower MW proteinaeses or be distinct large proteinase species.

Another aim of the recent investigations was to determine whether or not extensive differences occurred in response to culture mode i.e. shake or stationary cultures. It was found in the course of the experiments that various bands were produced by strains at different times in either culture mode, indicating that this parameter may not be crucial to the production of a particular protease or group of proteases. It was also noted that there was no significant differences between the number of bands produced in SH and ST culture modes overall.

As it was not possible to induce arthroconidial growth morphologies in the dermatophyte cultures or any morphological changes in the non-dermatophyte cultures by the techniques used, it is difficult to asses the signifigance of arthrospore production in relation to proteolytic enzyme production.

The investigations also examined the possibility that a band or group of bands may have been commonly produced by different strains of a particular species, or a particular group of bands may have been more commonly observed in one genus as opposed to another, perhaps contributing to the pathogenicity of the genus.

Examination of various aspects of band production did not reveal any bands common to one genus and not to another, with only two exceptions, all the species examined produced an assortment of bands of differing sizes at various times during the incubation periods.

The exceptions seen were *T. terrestre* and *C. keratinophilum*, the former producing only 1 band <30 Kda over the entire experimental period and the later producing only 3 band <30 Kda.

Overall, it was seen that those dermatophytic species which are commonly considered almost exclusively geophilic, only rarely causing infection in man or animals, more frequently produced the larger MW (>150 Kda) bands *in vitro* compared to those which are commonly reported as causing human disease. In contrast among the non-dermatophytic species, wide varieties of bands were seen, including some in the very small <10 Kda range. These differences could well be attributed to the differing host specifities of the species involved and enable the "opportunists" to grow on a wide variety of substrates.

Over the experimental period it was seen that two bands, of 23 and 21 Kda each were seen in all except two of the strains examined. These were *T. terrestre* and *C. keratinophylum*, which, as previously noted, produced bands predominantly in the larger MW range. Indeed, both of these strains generally demonstrated poor colony vigour using wool media and were deleted from further examination.

The large number of smaller proteases found in these studies supports the findings of other investigators. Characterisation of a number of these proteases has revealed that many of the low MW proteases are serine-proteases (Palmer 1995), generally found in the 18.5 to 35 Kda, although larger enzymes have been reported (North, 1982). Assuming serine proteases are critical in fungal pathogenesis, their small size would allow diffusion into the lower epidermis and dermis causing inflammation.

This view is supported by Minocha *et al.*,(1972) who noted observing dermo-epidermal separation and spongiosis when fungal extracts were injected intradermally into excised human skin. It may be that the ability of an opportunist to produce a particular proteinase or group of proteinases will determine its ability to survive on a living host.

During the examination of the effect of time on gelatinase production, it was noted that in only a few cases were the gelatinases which were expressed at seven days expressed throughout the age of the culture e.g.*M. canis* was seen to express a 125 Kda protease throughout the 97 gel runs and *D. chlamydosporium* strain 8 expressed larger MW proteases through the course of the trials. There was also a general decrease in the number of proteases detected towards the end of the culture period.

It has been suggested (Samdani *et al.*,1995) that this could be due to the contribution of autolysing hyphae at this time or a general expiry of the culture due to the limitation of the culture conditions e.g. space for growth versus the expiry of available nutrient sources and each strain of a particular species is likely to react differently to a prescribed set of conditions.

The present investigations have revealed an array of gelatinolytic enzymes which seem to be variable in their production and detection. Only several trends can be discerned in relation to commonality between strains, species and genera and to their significance in the ability to utilise keratinous substrates and/or cause disease.

Attempts to predict the behaviour patterns of these fungi via the number and type of consistently produced proteolytic enzymes is proving difficult due to the large number of variables involved with *in vivo* situations which simply cannot be reproduced *in vitro.*

A number of authors have also reported the production of multiple proteinases in other dermatophytes, such as *T. rubrum* and *T. mentagrophytes* (Meevootisom and Niederprum, 1979, Sanyal *et al.*, 1985, Ashai *et al.*, 1985,Apodaca and McKerrow 1989a,1989b,1990). Apodaca and McKerrow (1989a) have also suggested that dermatophytes express certain proteinases in response to different levels of sulphur, phosphorus, carbon and nitrogen. Papani *et al* (1996) found that *M. canis* isolates are capable of producing a variety of -

extracellular enzymes with producing different patterns in SDS-PAGE gels. They have suggested that the enzyme patterns of dermatophytes may be influenced by nutritional conditions.

These findings suggest that band patterns in themelves are not predictable and the pattern of enzyme expression may be influenced by a number of factors e.g. the concentration of gelatinases in the culture medium, the sensitivity of the SDS-PAGE technique, or the expression of specific proteolytic enzymes by the fungus in response to the availability of certain peptides and/or amino acids in the medium.

Further work in this area could be useful, particularly as host-parasite relations are dynamic and not an area which can be standardised.

It should also be noted that substrate-SDS-PAGE gels cannot be relied upon to produce accurate size estimations as demonstrated by Apodaca & McKerrow (1989a) who found when they purified an unreduced 27 Kda proteinase from *T. rubrum*, under reducing condition using standard SDS-PAGE, this proteinase was found to be 44 Kda in size. This size difference was considered to be mediated by intrachain disulphide bonds. However, there were clearly differences between MW of proteases found in the native form to those found partially denatured.

Therefore, each strain of a particular species is likely to react differently to a prescibed set of conditions.

It would be appropriate for more work to be carried out into the area of proteolytic enzyme production by various fungal species, particularly in light of emerging groups displaying opportunistic tendencies not previously noted.

CONCLUSION

The findings presented in this thesis confirm that animals and the soil are still important reserviours of potentially pathogenic fungi. Various potential opportunistic pathogens e.g. *Microsporum, Trichophyton, Aphanoascus* and *Scopulariopsis* all isolated from the above sources in this study.

All isolated species were readily identified on the basis of morphological observations as stated in the literature utilised. It is postulated that shake cultures of various dermatophytes more closely resemble the arthroconidial *in vivo* growth morphology, the only evidence seen of this was in the *M. gypseum* cultures which did on occasion resemble the morphology described in the literature.

However, in conclusion, the combined observations of gross colony morphology and microscopy in the identification of fungal species are still most efficient identification methods in terms of basic research.

Among the various observations carried out in this study, measurements of pH levels within culture vessels confirmed the work of earlier investigators of this parameter in relation to actual activity within the culture vessel being a measure of fungal activity.

Of the two gelatin hydrolysis methods used for rapid detection of proteolytic activity, the gelatin-disc method was most satisfactory with good results available from between 2 and 24 hours incubation with each culture medium. The glelatin hydrolysis plate method was not found to be of value in terms of proteolytic enzyme production however, was useful in terms of fungal growth and activity.

The use of lactrimel media in this study had a twofold purpose, that of re-establishing typical gross and microscopic fungal colonies and re-establishing proteolytic enzyme production in those species where this ability appeared reduced. This medium produced excellent results in both respects with an overall increase in proteolytic enzyme production of ~80% and a complete return to typical gross and microscopic morphologies were seen.

Gelatin/SDS-PAGE studies were carried out in order to establish whether or not a band or group of bands could be found in common to all species examined, either on the basis of genus or habitat. Two bands were seen as common to all but two of the species examined, at 21 and 23 Kda, although some other similarities seen, no others were seen as frequently.

There were no significant differences noted in the number of bands produced in either shake or stationary cultures, however, it was not also not possible to induce the 'arthroconidial' growth morphology in cultures for the duration of the SDS-PAGE trials therefore this it is not possible to state with certainty that shake cultures are or are not more effective for the production of proteolytic enzymes.

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APPENDIX A: BSW media (SeeResults 3.5)

Bands Produced by: *M. canis* **Strain/s:** 1, 3 & A

Media	Mode	Day	Kda >150	Kda 100-150	Kda 50-100	Kda 20-50	Kda < 20	Total Bands
AEOBSW	SH	7	173 151	109		21 19		5
		14	151		93, 85, 74 65, 52	23, 21		8
		21	151		93, 85	23, 21	17	6
		28	151		93	30, 25	10	5
						Total	bands	24
	ST	7	200,162*	134*		21	19	5
		14	165, 151	109		23, 21		5
		21	151	125,	85	25, 23, 21	17, 10	8
		28		125	85	30,25, 23, 21	10	7
						Total	bands	25
EOBSW 96	SH	7	190			23,21,	12	4
		14	190	100		23, 21	12	5
		21	151		83, 75	23, 21	10	6
		28		140		23, 21	10	4
						Total	bands	19
	ST	7	151			25, 23		3
		14	224, 190			25, 23	10	5
		21				25,23, 19	10	4
		28	151			30,25, 23	10	5
						Total	bands	17
EOBSW 97	SH	7		125	87,77,74	21	12	6
		14		125	74, 55	25, 23, 21	17	7
		21		145	70	25, 23	17	5
		28		145,125	70	25,23	17	6
						Total	bands	24
	ST	7		125		25, 23	17	4
		14		125	85, 74	25, 23		5
		21		125	85,70,65	25,23	17	7
		28		125	85,70	25, 23	17	6
						Total	bands	22

* Strain 3

Bands Produced by: *M. cookei* **Strain:** 6

Media	Mode	Day	Kda >150	Kda 100-150	Kda 50-100	Kda 20-50	Kda < 20	Total Bands
AEOBSW	SH	7				40,24		2
		14				57,23		2
		21	151		100,66,55	40,25,24 23,21	19,16, 14,12	13
		28	151		100,66,55	48,40,38 ,33,29,2 0	19,17,15, 12,11	15
						Total	bands	32
	ST	7	181			31,21		3
		14	200		65,57	45		4
		21	170		63	24,21		4
		28	165			48,43		3
						Total	bands	14
EOBSW 96	SH	7	165			30		2
		14	165			30	10	3
		21						0
		28						0
						Total	bands	5
	ST	7						0
		14	223, 170			20		3
		21						0
		28						0
						Total	bands	3

Bands Produced by: *M.cookei* **Strain:** K

Media	Mode	Day	Kda >150	Kda 100-150	Kda 50-100	Kda 20-50	Kda < 20	Total Bands
AEOBSW	SH	7				24,	19,18 17,17.5	5
		14			57		17,16	3
		21						0
		28						0
						Total	bands	8
	ST	7	200			31,21		3
		14	200					1
		21	170			22	18	3
		28	165	128		48,43		4
						Total	bands	11
EOBSW 96	SH	7	165			30		2
		14	165			30	10	3
		21			77	30		2
		28	165				10	2
						Total	bands	9
	ST	7	180			40,37		3
		14	223,170			20		3
		21				40,25, 22		3
		28	170	140		40,30		4
						Total	bands	13
EOBSW 97	SH	7				25,23.5 23		3
		14				25,23 22,20	19,18 17,16	8
		21				25,23	19,18 17	5
		28			74	30,25 23,20	19,18, 17,16	9
						Total	bands	25
	ST	7				40		1
		14				25,23 22		3
		21		125		30	17	3
		28	180		70	34,30 21	17	6
						Total	bands	13

Bands Produced by: *M. gypseum* **Strain:** B

Media	Mode	Day	Kda >150	Kda 100-150	Kda 50-100	Kda 20-50	Kda < 20	Total Bands
AEOBSW	SH	7				20	15	2
		14				23,21		2
		21	178			23,21	15	4
		28		125		40,38		3
						Total	bands	11
	ST	7	200,195			23,21	19,15	6
		14	200,195			23,21		4
		21		131,102		23	19	4
		28		145,100		23,21		4
						Total	bands	18
EOBSW 96	SH	7				36,28 26		3
		14					19,16	2
		21				28,23		2
		28	170			30		2
						Total	bands	9
	ST	7				30,23		2
		14		130	93	23,21		4
		21			100,70	35,30 23,21		6
		28		117	100,90	23,21		5
						Total	bands	17
EOBSW 97	SH	7				21	19,10	3
		14				23		1
		21	200			28,23		3
		28			74	31,23		3
						Total	bands	10
	ST	7		145		23		2
		14				23,21		2
		21			70	23, 21		3
		28	200			48,27 21		4
						Total	bands	11

Bands Produced by: *M. nanum* **Strain:** L

Media	Mode	Day	Kda >150	Kda 100-150	Kda 50-100	Kda 20-50	Kda < 20	Total Bands
AEOBSW	SH	7						
		14				23,21	19	3
		21	200			23,21	19,18	5
		28		125		30,27	11	4
						Total	bands	12
	ST	7	200	109				2
		14						0
		21	200			23,21	19	4
		28	190			40,37, 32,27	11	6
						Total	bands	12
EOBSW 96	SH	7		150	98	29,27, 25		5
		14	160	140	90		16,10	5
		21	190	140				2
		28						0
						Total	bands	12
	ST	7						0
		14	200,190,170				16,15	5
		21					16,11	2
		28		115	95		10	3
						Total	bands	10

Bands Produced by: *T.ajelloi* **Strain:** E

Media	Mode	Day	Kda >150	Kda 100-150	Kda 50-100	Kda 20-50	Kda < 20	Total Bands
AEOBSW	SH	7	175			20		2
		14	175			20		2
		21				20		1
		28			100	30		2
						Total	bands	7
	ST	7	>200, 200					2
		14	>200, 200					2
		21						0
		28	165					1
						Total	bands	5
EOBSW 96	SH	7	200			22		2
		14	170	112				2
		21	170	112				2
		28	170		87	30		3
						Total	bands	9
	ST	7	200					1
		14	200, 170		63	30		4
		21	200					1
		28						0
						Total	bands	6
EOBSW 97	SH	7	200	145	87, 77	23,22 21		7
		14	>200, 200		63	23, 22		5
		21	>200, 200					2
		28	>200, 200			23, 22		4
						Total	bands	18
	ST	7	>200	145, 108	67, 63	45, 24		7
		14	180	145	63			3
		21	180		90			2
		28	200	145, 100	79	28, 25	17	7
						Total	bands	19

Bands Produced by: *T. mentagrophytes var mentagrophytes* **Strain:** D

Media	Mode	Day	Kda >150	Kda 100-150	Kda 50-100	Kda 20-50	Kda < 20	Total Bands
AEOBSW	SH	7		125	80	40, 20	19	5
		14			75, 63	25, 23		4
		21			95	30	18, 8	4
		28		125	80	40	8	4
						Total	bands	17
	ST	7	>200,160			45, 40 32, 24 20	19	8
		14	170	140	60	40,24 23,22		7
		21			85	40,24		3
		28			85	23	8	3
						Total	bands	21
EOBSW 96	SH	7			85			1
		14		100		20	16, 7	4
		21	>200	150	90,78	50,37, 23		7
		28				21	8	2
						Total	bands	14
	ST	7	165			48, 32		3
		14				47	18, 7	3
		21				47, 28		2
		28			75	32	7	3
						Total	bands	11
EOBSW 97	SH	7	180		87, 79, 72	22, 20		6
		14			87	32, 22, 20		4
		21		141		22, 20		3
		28		114	70, 55	47, 28		5
						Total	bands	18
	ST	7	>200,180	107	68, 63	45, 24		7
		14	180	145	74, 68	24, 23, 22		7
		21	180	145	90	28		4
		28	180	145	90, 68, 51	38, 30		7
						Total	bands	25

Bands Produced by: *T.erinacei* **Strain:** M

Media	Mode	Day	Kda >150	Kda 100-150	Kda 50-100	Kda 20-50	Kda < 20	Total Bands
AEOBSW	SH	7				23, 20	16,15	4
		14			58	23		2
		21			66	23, 21		3
		28				36	9	2
						Total	bands	11
	ST	7						0
		14			58	23		2
		21	158		66	23, 21		4
		28				33, 24, 20		3
						Total	bands	9
EOBSW 96	SH	7						0
		14	190	112	73		18, 16	5
		21	>200	150	78, 67	26, 23		6
		28				36, 22	18, 10	4
						Total	bands	15
	ST	7	158			29		2
		14			65		18, 9	3
		21	190					1
		28				36, 22	9	3
						Total	bands	9

Bands Produced by: *T. terrestre.* **Strain:** J

Media	Mode	Day	Kda >150	Kda 100-150	Kda 50-100	Kda 20-50	Kda < 20	Total Bands
AEOBSW	SH	7		100		24		2
		14	200	100				2
		21	190	125, 100				3
		28		100	80, 60			3
						Total	bands	10
	ST	7	160		83			2
		14		140	75, 70			3
		21		140	70			2
		28		125	100, 52			3
						Total	bands	10
EOBSW 96	SH	7						0
		14		140				1
		21	200	150, 115	75			4
		28		120	64			2
						Total	bands	7
	ST	7	160					1
		14	200	130	77, 65			4
		21	160		60			2
		28			65			1
						Total	bands	8

Bands Produced by: *C. keratinophilum* **Strain:** I

Media	Mode	Day	Kda >150	Kda 100-150	Kda 50-100	Kda 20-50	Kda < 20	Total Bands
AEOBSW	SH	7						0
		14	200, 165					2
		21	186			31		2
		28	200, 165					2
						Total	bands	6
	ST	7	186,160, 160					3
		14	200,190, 180					3
		21	200					1
		28	200					1
						Total	bands	8
EOBSW 96	SH	7						0
		14					19	1
		21						0
		28		134		22		2
						Total	bands	3
	ST	7	200, 165					2
		14	180, 165	105				3
		21						0
		28						0
						Total	bands	5

Bands Produced by: *A terreus* **Strain:** H

Media	Mode	Day	Kda >150	Kda 100-150	Kda 50-100	Kda 20-50	Kda < 20	Total Bands
AEOBSW	SH	7				20	19, 18	3
		14			57	24, 20	19	4
		21				30, 24	19, 18	4
		28			100	48		2
						Total	bands	13
	ST	7				22, 20	19, 18	4
		14				28, 23, 20	19,18, 16	6
		21	>200			23, 20	19, 16	5
		28				35	19	2
						Total	bands	17
EOBSW 96	SH	7						0
		14					18, 15 14, 12	4
		21				30	19,16, 15	4
		28			98	21	16,15, 14	5
						Total	bands	13
	ST	7			79, 77			2
		14				24, 21, 20	15	4
		21					19, 16,15, 14,13	5
		28				30, 21	17, 15	4
						Total	bands	15
EOBSW 97	SH	7	181		77	25	16	4
		14		138	80	40, 25, 24		5
		21	200		70	28		3
		28			70	36, 34		3
						Total	bands	15
	ST	7			80, 77			2
		14			63	25, 22, 21		4
		21		145	75	30, 24, 23		5
		28			63, 55	23, 22, 21		5
						Total	bands	16

Bands Produced by: *S. brevicaulis* **Strain:** F

Media	Mode	Day	Kda <150	Kda 100-150	Kda 50-100	Kda 20-50	Kda < 20	Total Bands
AEOBSW	SH	7					10	1
		14				20	6	2
		21				20	6	2
		28				35, 20	8, 7	4
						Total	bands	9
	ST	7					10	1
		14			100, 70	23, 20	19, 6	6
		21			100, 75	23, 20	19, 8, 7	7
		28				35, 20	19, 8, 7	5
						Total	bands	19
EOBSW 96	SH	7				31, 25	19, 14, 7	5
		14					17, 15, 14, 7, 5	5
		21			83, 70	27	7	4
		28			75	33, 21	7	4
						Total	bands	18
	ST	7				36, 23	7	3
		14					18, 16, 14, 7,6	5
		21					19, 17, 15, 7	4
		28					19	1
						Total	bands	13
EOBSW 97	SH	7			57	23, 20	8	4
		14			85	39, 23, 22, 21	14, 12	7
		21			90	38	14	3
		28			90, 70, 63, 56	34, 32, 30, 25	12	9
						Total	bands	23
	ST	7	160	125		24	9, 8, 6	6
		14			100, 52	23, 22, 21	12	6
		21				25	14, 13	3
		28				23, 22, 21	14, 12	5
						Total	bands	20

Bands Produced by: *D. chlamydosporum* **Strain:** 7

Media	Mode	Day	Kda >150	Kda 100-150	Kda 50-100	Kda 20-50	Kda < 20	Total Bands
AEOBSW	SH	7			100	20	19, 18	4
		14			84, 65	50	18, 10	5
		21			95, 75		14	3
		28	>200				8	2
						Total	bands	14
	ST	7			84, 75		19, 18, 17	5
		14			84, 65 60	50,24, 23,21	20, 18, 17, 15, 14	12
		21			84, 75	21	18, 17, 14	6
		28			65, 60	30, 26, 23		5
						Total	bands	28
EOBSW 96	SH	7						0
		14		130			16	2
		21			100	21		2
		28					16	1
						Total	bands	5
	ST	7	190		62			2
		14	190		91		16, 12	4
		21	190				17, 13, 12, 11	5
		28		150			13, 12, 11	4
						Total	bands	15

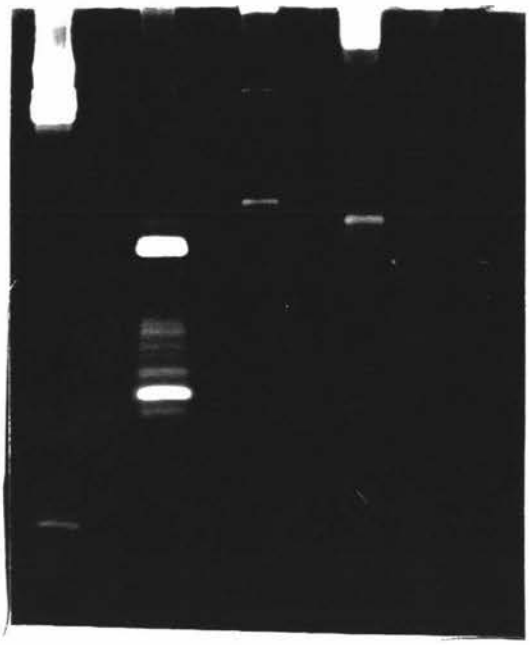
Bands Produced by: *D. chlamydosporum* **Strain:** 8

Media	Mode	Day	Kda >150	Kda 100-150	Kda 50-100	Kda 20-50	Kda < 20	Total Bands
AEOBSW	SH	7		150		23, 21	20, 19, 18, 17, 16	8
		14			84, 65	22	19, 14	5
		21			95		14	2
		28	190	125	100	33, 30		5
						Total	bands	20
	ST	7	180		100, 95, 55	25	20, 19, 18, 16	9
		14			84, 65, 60, 50	24, 23, 21	20, 16, 14	10
		21			85, 75	21	16, 14	5
		28		125		30	18, 14	4
						Total	bands	28
EOBSW 96	SH	7		150		20	17	3
		14		150, 120	63		17, 9	5
		21		104	63	20	17	4
		28		150		22, 21, 20	19, 14	6
						Total	bands	18
	ST	7	190		60	48	15	4
		14	160			43, 25	17, 9	5
		21				20	13, 12	3
		28				21	13, 12, 11	4
						Total	bands	16
EOBSW 97	SH	7	>200			21	16	3
		14	>200		63	21		3
		21	>200	112				2
		28						0
						Total	bands	8
	ST	7	>200	144				2
		14		150	90, 60	25, 23, 20	18, 17	8
		21			90	40, 35, 26, 21, 20		6
		28		150	90	20		3
						Total	bands	19

APPENDIX B

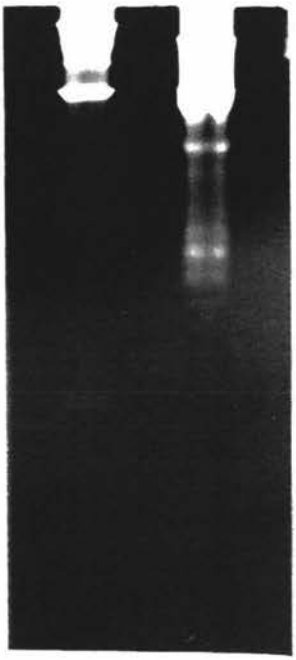
Photographic evidence of band production by various species in BSW.

Lane 1 2 3 4



BSW SH cultures
1 = *S. brevicaulis*
2 = *M. cookei*
3 = *M. gypseum*
4 = *M. nanum*

Lane 1 2

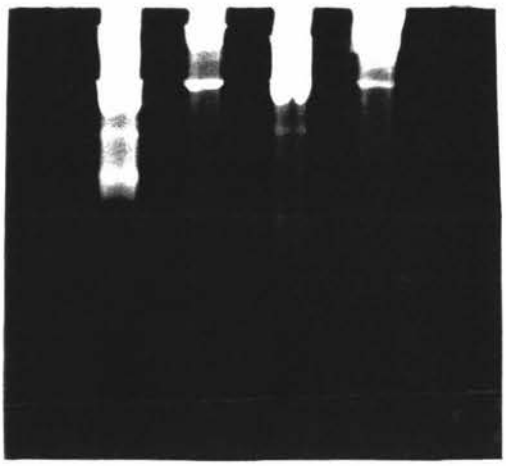


BSW SH cultures

1 = *T. mentagrophytes*
2 = *D. chlamydosporium*

Lane 1 2 3 4 BSW SH cultures

1 = *T. mentagrophytes*
2 = *T. ajelloi*
3 = *T. erinacei*
4 = *T. terrestre*



Lane 1 *A. terreus*