IN VITRO MODELS FOR ASSESSING THE PATHOGENICITY OF *MALASSEZIA* YEASTS

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ABSTRACT

The basidiomycetous yeast *Malassezia* has been linked to a number of disease states such as seborrhoeic dermatitis, dandruff and pityriasis versicolor. Much confusion has arisen as to its role in these disease states as this fungus is found inhabiting the stratum corneum of approximately 90% of the human adult population. *Malassezia* yeasts are lipophilic organisms, some species showing a specific requirement for long chain fatty acids for *in vitro* culture.

The object of the experiments undertaken in this study was to elucidate the role of *Malassezia* yeasts in dandruff and seborrhoeic dermatitis. *In vitro* culture was used to ascertain their potentiality to form hyphal filaments and to study these filaments and their role in pathogenicity pertaining to dandruff and seborrhoeic dermatitis. A review of novel and existing methods of inducing filament production in *Malassezia* yeasts was undertaken. Using scanning electron microscopy (SEM) a detailed study of hyphal length and structure was carried out under various conditions. In each case, filaments were approximately 30-60μm in length with an irregular shape, in comparison to the short filaments normally isolated from pityriasis versicolor lesions. Filaments were also observed to extend from various points on the parent cell surface. The production of phialospores was also observed, in which a filament extends from the parent cell and a phialide is extruded from the opposite end of the filament. In Dorn's filamentation broth [63] filaments were observed to form in *M. furfur* isolates at 29°C but not at 37°C. A link between divalent cations and glycine were also highlighted in these cultures.

Squalene, an ergosterol (primary membrane sterol) precursor, and cholesterol were also shown to induce the production of filaments in various *Malassezia* isolates, on stripped stratum corneum D-squame biopsies. This provides a link between membrane sterol composition, membrane fluidity and hyphal production. As filament production was difficult and not universal for all *Malassezia* isolates , linked with the infrequent isolation of *Malassezia* filaments in seborrhoeic dermatitis, it was concluded that although this structure is observed to aid deep penetration of the stratum corneum in pityriasis versicolor lesions, it is unnecessary for pathogenicity in seborrhoeic dermatitis .

As well as examining filament structure under scanning electron microscopy, the formation of an extracellular capsular-like coating was observed under all conditions examined, covering clumps of yeast cells. It is hypothesised that this coating may aid *Malassezia* pathogenicity by keeping the yeast in contact with the host surfaces and by providing a protective layer against destruction.

Initial colonisation events of *M. furfur* were also investigated using a living skin equivalent model for growth. Living skin equivalents were constructed from a synthetic dermal component formed from type one collagen contracted by the presence of foreskin dermal fibroblasts, and an epidermal component formed by seeding neonatal foreskin keratinocytes onto contracted collagen lattices. A stratified epidermis was formed by incubation of the models in submerged culture followed by incubation at an air-liquid interface. Assessment of fungal growth and invasion was by light and scanning electron microscopy. Yeast cells were inoculated onto LSEs,

which were incubated in CO_2-independent media at 37°C for variable lengths of time. Viability counts of *M. furfur* were determined by a method of washing and serial dilution. Yeast cells had retained their viability and increased in number approximately two fold over a four day period of incubation. Yeast-to-hyphal transition was not achieved in this model. Random destruction of the uppermost layers of the stratum corneum was observed in the presence of *M. furfur*, which bore similarities to the desquamation observed in dandruff lesions. Penetration of the stratum corneum was confined to the most superficial layers of the stratum corneum with no evidence of yeast cells or filaments in the deeper layers of the stratum corneum.

In comparison to the skin biopsy D-squame method, the LSE model was found to provide a model with a fully formed epidermis, which is noninfected, by resident microflora. The use of LSEs for studying fungal inhabitation of the stratum corneum opened the possibilities of using such models in the examination of topical treatments and antifungals in a more *in vivo*-like model. This was the first study in which living skin equivalents have been used as an *in vitro* model to assess the pathogenicity of *M. furfur*. The results show close similarities between the growth of the yeast phase of *M. furfur* on an LSE and growth *in vivo* in mild cases of seborrhoeic dermatitis.. It was hypothesised that lack of deeper invasion may be due to lack of sebaceous gland lipids and therefore a different lipid distribution *in vivo* than *in vitro* of lipids beneath the skin surface. Also, invasion of the skin models was also hindered by the inability of the yeasts to form filaments, so deeper penetration could not occur by the methods observed in pityriasis versicolor. However, as these models provide a growth substrate for *Malassezia* yeasts this allows the possiblity of LSEs being used to examine the

manipulation of *M. furfur* growth and viability by the addition of exogenous material. Another benefit is that use of LSEs may lessen the need for use of animal models for the investigation of cutaneous infections, although it is hypothesised that its main use will most likely be found in the screening of antifungal drugs in a predictive manner, under carefully monitored conditions.

Because *Malassezia* yeasts are known to inhabit the pilosebaceous unit of a high percentage of healthy individuals and are closely associated with conditions known to affect hair follicles, a hair shaft model was developed to assess the effects of growth and form of *Malassezia* yeasts. The aim was to ascertain whether or not *Malassezia* yeasts had a predilection for a specific component of the hair follicle.

Intact follicles were plucked from the heads of healthy volunteers, microdissected into specific hair shaft components and infected with *M. furfur in vitro* in PBS. To assess growth and viability under these conditions, viability counts were carried out at the beginning and end of incubation. After three days incubation at 29°C, it was observed that the nonemerged shaft had the greatest effect on *M. furfur* viability for all the donors examined. This shaft region lies essentially within a pool of sebum, as it lies directly above the sebaceous gland therefore in the most lipid rich region of the hair shaft, and may provide a reservoir for the lipophilic microflora of the pilosebaceous unit. Under SEM, *M. furfur* also showed a predilection for colonisation of the nonemerged shaft, reinforcing the idea of this shaft region as a reservoir for *Malassezia* yeasts. In conclusion, this chapter showed that *M. furfur* and *M. sympodialis* have a predilection for the lipid rich area of the hair shaft, in particular the nonemerged, fully keratinised shaft, though this may be effected by hair type.

Although this work did not indicate any specific pathogenic mechanism, it supported the existing evidence for a major host role in *Malassezia*-related diseases and suggests that investigations into the role of occlusion and sebum excretion rate are required to elucidate more information on the pathogenicity of *Malassezia*-related infections.

The effect of extracted sebum on the growth of *M. furfur* was also examined in this study. Sebum was collected from the foreheads of healthy volunteers and extracted using hexane as the solvent. Sebum, which was resuspended in PBS by sonication was incapable of stimulating growth of *M. furfur*. It was observed however that pooled sebum samples which were concentrated by centrifugation, were capable of stimulating growth of *M. furfur*, increasing the number of viable yeast cells per millilitre by two-to-four-fold over 72 hours. When these samples were serially diluted it was observed that the 10^{-1} dilution (approximating a 10% sebum suspension) was still capable of stimulating *Malassezia* growth, although the 10^{-2} dilution (approximating a 1.0% sebum suspension) did not stimulate growth. It is hypothesised that a similar effect may be observed at the stratum corneum surface, when sebum is dispersed in surface sweat and less capable of stimulating *Malassezia* growth.

Because overproliferation of *M. furfur* growth was not observed in this model, it was suggested that other factors such as bacterial lipases and sebaceous duct lipases as well as other stratum corneum surface components play a role in the stimulation of *M. furfur* growth *in vitro*.

When concentrated sebum was added to plucked anagen hair shafts, viable growth of *M. furfur* was observed over 72 hours at 29°C. The addition of squalene to

anagen hair shafts did not stimulate growth although addition of oleic acid to anagen hair shafts did stimulate *M. furfur* growth. Growth in the presence of hair shaft plus concentrated sebum stimulated *M. furfur* growth better than either model alone suggesting that both hair shaft factors play a role in growth and maintenance of *M. furfur* at the scalp surface. Concentrated sebum added to stripped stratum corneum samples was also observed to stimulate *M. furfur* growth in an additive manner although overproliferation of *M. furfur* was not observed. Again, this suggested that a combination of host and environmental factors are involved in the overproliferation of *M. furfur in vivo*.

Serial strippings of the stratum corneum were infected with *M. furfur* to compare by scanning electron microscopy growth and morphology of *M. furfur* at various levels of the stratum corneum. *M. furfur* showed pleomorphic tendencies at the stratum corneum levels. A low frequency of hyphal production was observed in control, unsupplemented stripped stratum corneum, which were observed to penetrate under corneocyte surfaces and bury into keratinised material. There was no difference in frequency of filament production between the various levels of the stratum corneum. It is suggested that a starvation mechanism may be one of the triggers for filament formation. From this study it was concluded that concentrated sebum and stratum corneum may enhance the growth of *M. furfur*. Stratum corneum and hair shaft factors are most likely to be associated with lipids and fatty acids present and the action of endogenous bacterial population.

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DEDICATION

To my mother and father, in honour of their love and support.

Declaration

I hereby declare that this thesis embodies the results of my own original work, that it has been composed by myself and has not been submitted for consideration for any other degree in this or any other University.

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1. CHAPTER 1: GENERAL INTRODUCTION

1.1 HISTORY OF *MALASSEZIA*: MORPHOLOGIC AND GENETIC CONSIDERATIONS

The basidiomycetous genus *Malassezia* comprises globose to ellipsoidal, unipolar budding yeasts with a specific lipophilic requirement for most species [115]. Until recently, these yeasts have been classified in the heterogeneous family, Cryptococcaceae by Barnett [12] and Kreger van Rij [129]. The basidiomycetous nature of these fungi is indicated by a positive diazonium blue B (DBB) staining reaction, ability to hydrolyse urea, a lamellar cell wall structure and the ability to resist cell wall lysis by β-(1-3)-D- glucanase [115].

Since the first recognition of the yeasts in 1846 [70] the classification and taxonomy of these yeasts have been dogged with much controversy and confusion. Early workers such as Robin [191] were the first to link fungi with the disease pityriasis versicolor by isolating mycelial elements in skin scales from lesional sites of patients with pityriasis versicolor. In 1873, Rivolta linked the organism to psoriasis when he visualised the organism from flakes of crusty skin observed in his beard [189].

A year later, Malassez [143] isolated these organisms from a number of skin conditions and considered them to be a cause of scalp scaling. The name *Malassezia furfur* was proposed by Baillon in 1889 in recognition of the work of Malassez, the term "furfur" being used to describe the characteristic scaling or furfuraceous nature

of pityriasis versicolor [9]. Although hyphal filaments were observed earlier in pityriasis versicolor lesions, Sabouraud in 1904 [195] was the first to consider a link between the yeast cells and hyphal elements in pityriasis versicolor lesions and proposed the genus *Pityrosporum* to describe the yeast cells which he observed in the absence of hyphae in normal skin and scalp, the name being derived from the description "spore of the skin" [115].

Based on these observations, Kraus [128] concluded that the *Pityrosporum* yeast was the spore stage of the hyphal elements observed in pityriasis versicolor lesions. Credit was given to Castellani and Chalmers, who were the first to consistently culture the organisms from skin scales and proposed the name *Pityrosporum ovale* [40]. In 1925, Weidman discovered a non-obligatory lipophilic yeast on the skin of animals which he considered as being of the same genus as the organisms observed by Malassez, and proposed the name *Malassezia pachydermatis* [230]. This yeast is now known to be more commonly associated with animals than humans, in particular canines [113]. In canines it is known to be the causative organism of the disease otitis externa [1]. However, *M. pachydermatis* has also been associated with systemic infections in humans [132]. The term *P. orbiculare* was used in 1951 by Gordon to describe round yeast cells observed in pityriasis versicolor and normal skin, which he regarded as a separate entity from *P. ovale* [89]. A further species called *Malassezia sympodialis* was later discovered in 1993 by Simmons and Guého [208].

In *P. ovale* and *P. orbiculare* morphological instability had been noted by various authors [197]. Whereas some authors proposed that these two organisms were distinct entities which could co-exist in diseased and normal skin, others

believed that they represented two morphological forms of the one species that were directly interchangeable and represented different stages in the life cycle of the filamentous *M. furfur* [83]. These organisms have also been classified under the genus names *Microsporon* [191], *Cryptococcus*[189] *Saccharomyces* [25], *Monilia* [224] and *Dermatophyton* [62].

The confusion of taxonomy has most probably arisen from the fact that all early descriptions were based on microscopic observations because of the difficulties in culturing these organisms *in vitro*. Due to scientific progress, techniques for classifying micro-organisms are now more sophisticated and diverse. In the past two years the confusion surrounding the classification of these organisms has been clarified, and in 1996 a new taxonomic classification was proposed encompassing seven different species [97] and consequently, a new taxonomy for the genus *Malassezia* was published. This genus now comprises the species *M. furfur, M. sympodialis, M. obtusa, M. restricta, M. globosa, M. slooffiae* and *M. pachydermatis*. Using morphology, ultrastructure and physiology of *Malassezia* yeasts, the new classification of the genus *Malassezia* is as follows:

1. ***Malassezia furfur* (Robin) Baillon:** On Dixon's agar (see appendix), colonies are mat, smooth, umbonate or slightly folded with a soft friable texture. Yeast cells show pleomorphic tendencies with cylindrical cells being approximately (1.5 - 3.0) x (2.5 - 8.0)μm and spherical cells being (2.5-5.0)μm in diameter. This species buds from a characteristically broad base, with the ability to form filaments. Catalase, urease and Diazonium Blue B reactions are positive, and they can use

Tween 20, Tween 40, Tween 60 or Tween 80 as a sole lipid source *in vitro*. *M. furfur* has a GC content of 66.4 ± 0.3%, with an unknown teleomorphic state [97,98].

2. ***Malassezia pachydermatis* (Weidman) Dodge:** On glucose/peptone agar, colonies are mat, cream and convex or umbonate, with a texture similar to *M. furfur*. The yeast cells are small and ovoid (2.0-2.5)µm x (4.0-5.0)µm, with a prominent bud scar, buds forming from a broad base. Catalase reaction is variable, but urease and DBB reactions are positive. Tween 20 inhibits growth. *M. pachydermatis* has a GC content of 55.6 ± 0.2% with an unknown teleomorphic state [97,98].

3. ***Malassezia sympodialis* (Midgley):** On Dixon's agar, colonies are glistening, smooth and flat or slightly raised with a soft texture. Cells are oval to globose (1.5-2.5) x (2.5-6.0)µm with the bud base narrower than *M. furfur* and the parent cell. Characterised by repetitive or sympodial budding. Catalase, urease and DBB reactions are as for *M. furfur*. Tween 40, Tween 60 or Tween 80 can be used as a sole lipid source *in vitro* and cells have a GC content from 54.0-62.2±0.2% [97,98].

4. ***Malassezia globosa*:** Midgley, Guého & Guillot. Smooth, flat colonies with a sticky texture are observed on incubation on Dixon's agar. Yeast cells are large and cylindrical (1.5 - 2.0) x (4.0 - 6.0)µm, with buds forming on a broad base. Branching filaments may be observed. Catalase, urease and DBB reactions are as for *M. furfur* and require a mixed Tween lipid source for growth *in vitro*. Cells also have a GC content of 60.7% [97,98].

5. ***Malassezia obtusa*** : Midgley, Guillot & Guého: These colonies have characteristically rounded apices and are smooth and flat with a sticky texture on Dixon's agar. Yeast cells are large and cylindrical (1.5-2.0x 4.0-6.0μm). Filaments may occur at any point on the parent cell. Catalase, urease and DBB reactions are positive. Individual Tweens at concentrations of 0.1%-10% are unable to support growth on glucose-peptone agar. Cells have a GC content of 60.7% [97,98].

6. ***Malassezia restricta*** : Gueho, Guillot & Midgley:. Colonies are dull with variable edges and a hard brittle texture. Yeast cells are spherical or oval (1.5 - 2.0) x (2.5 - 4.0)μm with buds formed on a relatively narrow base. Catalase reaction does not occur, but urease and DBB reactions are positive. Tween 20, Tween 40, Tween 60 or Tween 80 are not capable of supporting growth as a sole lipid source *in vitro*. The GC content is 59.5% ± 0.1% with no teleomorphic state [97,98].

7. ***Malassezia sloofiae***: Midgely. Colonies are usually rough with grooves and a course texture on Dixon's agar, Yeast cells are short and cylindrical (1.0-2.0) x (1.5-4.0) μm and bud from a broad base. Catalase, urease and DBB reactions are all as for *M. furfur*. Tween 40 or Tween 60 may act as a sole lipid source and cells have a GC content of 68.7% ± 0.1% [97,98].

Easy diagnosis of pityriasis versicolor is accomplished using direct microscopy of skin scrapings from lesional sites in KOH mounts stained with calcofluor white for fluorescence microscopy or lactophenol cotton blue [115] by light microscopy. Short filaments in abundance should be observed in the presence of budding yeast

cells. Sellotape stripping of lesional sites may be stained and mounted on glass slides for microscopic examination and preservation.

Culture of the organisms may aid in diagnosis of seborrhoeic dermatitis and folliculitis. Skin scrapings may be seeded onto a number of *Malassezia*-promoting solid medium. Such medium include Dixon's agar [219], glucose yeast peptone agar supplemented with olive oil, Tween 80 and glycerolmonostearate (Gyp-2 agar), [74] or Leeming's medium, which consists of Gyp-2 agar supplemented with glycerol, ox-bile and whole fat cow's milk in place of olive oil [219]. Quantitative examination can be achieved by using wash and scrub techniques [82] and contact plates[133] as well as the Scotch tape method of Wikler *et al* [235].

1.2 CULTURAL REQUIREMENTS AND *IN VITRO* AND ULTRASTRUCTURAL CHARACTERISTICS

Apart from *M. pachydermatis* isolates, all *Malassezia* yeasts show an absolute dependency on the presence of long chain fatty acids in the culture medium [161]. Many studies have shown a blocked capacity for synthesis of fatty acids of chain length C12 - C16 [145, 205]. *Malassezia* species are normally associated with body and facial sites where there are numerous sebaceous glands. In such areas, growth is stimulated by the presence of sebum lipids and free fatty acids, due to the decomposition of keratinised material [162], and such lipids are therefore required for growth *in vitro*[161,74]. Both saturated and unsaturated fatty acids of suitable

chain length will stimulate growth *in vitro*[205]. The fatty acid composition of the organism reflects the fatty acids present in the culture medium, which suggests that fatty acids are non essential growth factors but may be required for membrane synthesis [161]. *Malassezia* yeasts will grow over a wide range of temperatures (21°C - 37 °C) and there is no evidence of their requiring another carbon source as well as the lipid source for growth *in vitro* [161]. Although few specific amino acids have been shown to enhance *M. furfur* growth, they are all thought to be non essential [19]. Since *Malassezia* yeasts possess the ability to use lipid substrates for growth, it suggested that there must be some enzymatic activity associated with the breakdown of these lipids. Thus far, Catterall *et al* [41] discovered a lipase in *P. orbiculare* which was capable of utilising Tween 80 *in vitro*, but appeared to have little effect *in vivo*. This lipase activity was then shown by Ran and co-workers to be associated with the insoluble fraction of the organism [181]. Work by Plotkin suggested that there were at least three lipases in *M. furfur* that could digest lipidic substrates [174]. However it is thought that *in vivo*, *Malassezia* and phospholipase activity are non important for the production of free fatty acids due to the role that other members of the cutaneous microflora play in the break down of lipids at the skin surface [41].

Lipoxygenase activity has also been shown in *Malassezia* yeasts. It is known that *in vitro*, *M. furfur* oxidises unsaturated fatty acids, unsaturated triglycerides and cholesterol [158]. However, there is no evidence of lipoxygenase activity *in vitro*. It has been hypothesised however that increased levels of lipoperoxides may be demonstrated responsible for some of the pathological changes associated with

pityriasis versicolor as increased levels are observed at lesional but not non-lesional skin sites [158].

Structural variability of *M. furfur* appears to be associated with the development of the organism *in vivo* and *in vitro*. In 1994, Mittag conducted *in vitro* examinations of *M. furfur* ploidy and a structural investigation of the yeast cell envelope. *M. furfur* yeast cells were found to be characterised by a well circumscribed nucleus with a clearly visible nuclear envelope with nuclear pores. There was an apparent association of two large vacuoles with the nucleus. Small and thin cells were associated with the exponential growth phase. A large variation in yeast cell size was observed in skin scale cultures from cases of pityriasis versicolor. The majority of *Malassezia* cells observed were considered haploid in relation to their surface/volume quotient, although large oval cells showed a surface/volume quotient indicative of a diploid genome. The variable ploidy was thought to dictate the ability in individual yeast cells to utilise certain amino acids and why the smaller oval and cylindrical cells are easily cultivated *in vitro* and more frequently isolated from saprophytic growth on human skin [139].

An electronmicrographic study of the cell membrane showed a thin outer lamellar layer. Electron dense material could be seen attaching to adjacent cells, suggesting that the lamellar layer was important in the attachment process in *Malassezia* infections. The lamellar layer also appeared to contain lipid components, making the overall membrane appearance suggestive of a capsular layer similar to that observed in *C.albicans*. The cell wall appeared to be separated into two major

compartments, with both layers showing a lamellated structure, with protein being concentrated in the outer part of the cell wall [138].

1.3 SEROLOGY

A serological relationship between cultural and micromorphological variants of *M. furfur* have been demonstrated in various studies [81, 217]. Takahaski *et al* associated three distinct groups of soluble antigens in various micromorphological variants [216]. Cunningham *et al* designated three distinct serovars of *M. furfur* as A, B and C [54]. Using an improved medium for the primary isolation of *M. furfur*, Cunningham *et al* were able to demonstrate that these serovars corresponded to three culturally stable variants. These serovars are now classified as *M. sympodialis* (serovar A), *M. globosa* (serovar B) and *M. restricta* (serovar C) as designated by Gueho *et al* [97].

1.4 *MALASSEZIA* RELATED INFECTIONS

1.4.1 SEBORRHOEIC DERMATITIS AND DANDRUFF

The disease state seborrhoeic dermatitis is viewed as a chronic condition with a predilection to cause lesions in sebaceous gland-rich regions of the skin such as the scalp, retroauricular region, auditory canals, nasolabial folds, trunk and intertrigines, and is characterised by redness and diffuse yellowish greasy scaling [115]. It is thought to have a higher prevalence in immunocompromised patients, in particular in humanimmunodeficiency virus-positive (HIV+) patients [149]. Dandruff, on the other hand, is confined to the scalp region and is regarded as a non inflammatory chronic condition with hyperkeratosis of the scalp [127]. Some investigators regarded seborrhoeic dermatitis and dandruff as being non-related separate clinical entities [127], while other groups regarded dandruff as being merely a mild form of scalp seborrhoeic dermatitis [83]. At present, the facts that a large number of *Malassezia* yeasts are involved in each case, and both conditions rapidly clear upon topical anti-*Malassezia* treatment, endorse the idea of a disease continuum between seborrhoeic dermatitis and dandruff.

There has always been much confusion associated with the actiology and diagnosis of seborrhoeic dermatitis and dandruff. Most of the evidence backing the role of *Malassezia* yeast in seborrhoeic dermatitis is indirect and mostly based upon the observations that upon treatment of seborrhoeic dermatitis with anti-*Malassezia* agents, there is a reduction in fungal load associated with skin scales and clinical improvement during and after treatment. For example, Gosse and Vanderwyke showed that when they removed the scalp microflora of dandruff sufferers with nystatin and neomycin, dandruff severity was reduced but upon recolonisation with a nystatin-resistant strain of *M. furfur*, the disease state could be reinduced [91]. Reduction in dandruff severity is also noted upon treatment with selenium sulphide

[86,138] zincpyrithione [147], and topical ketoconazole [37,84], all of which have in common antifungal properties.

Other investigators support a purely saprophytic role because no protective host response is provoked in clinically normal skin colonised by *Malassezia* yeasts, Also, because *Malassezia* yeasts are found in the skin of approximately 90% of normal individuals, it means that Koch's postulates cannot be fulfilled, casting doubt on an eitiological role for *Malassezia* yeasts in seborrhoeic dermatitis [190]. Leyden and coworkers also opposed the view of a role for *Malassezia* yeasts in dandruff stating that dandruff is not a disease but is merely an intensification of the physiologic process of desquamation and exogenous microbial factors, in particular *P. ovale* can be eliminated as factors in the pathogenesis of dandruff [138]. Reviewing the literature, Shuster [207] managed to show that the aspersions cast on the role of *Malassezia* yeast in seborrhoeic dermatitis and dandruff by Kligman's teams of coworkers [127,138] and by Imokawa *et al* [116] were based upon misinterpretation of data and poor experimental design [207] and upon one piece of negative evidence, where topical amphotericin was shown not to decrease dandruff even though the yeast population was decreased [207].

Due to the advent of successful anti-*Malassezia* treatments, Koch's postulates were finally fulfilled in that the yeast was present in both disease states, cleared up on treatment, and disease returned upon reinfection with *Malassezia* yeasts [91]. Ongoing work in this field is now focused on determining an exact role for *Malassezia* yeasts in seborrhoeic dermatitis, and their relationship with the host and host response.

1.4.2 INFANTILE SEBORHOEIC DERMATITIS (ISD)

Although seborrhoeic dermatitis is thought to be a post-pubertal disease, being associated with pilosebaceous gland excretions, there is evidence that a similar disease occurs in infants. Some investigators propose that ISD is a clinical variant of atopic dermatitis [175] other investigators proposed that it is a distinct entity. Although *Malassezia* yeasts may be found commonly in healthy adults, this is uncommon in infants, hence the difficulty to create a firm link between ISD and post -pubertal seborrhoeic dermatitis.

Infantile seborrhoeic dermatitis presents very early in life and may clear spontaneously within three months. It is thought that the onset of ISD coincides with the onset of neonatal "puberty" created by the influences of maternal and placental hormones. The skin lesions are red and scaly involving the scalp, flexures or napkin area [133].

Candida albicans has also been implicated in the aetiology of ISD [32] and *S. aureus* has been proposed to be a secondary invader [32]. In a study of ISD in 20 infants, *P. ovale* (*Malassezia*) was isolated from 18/20 infants with ISD and it was proposed that the similarities between endrocinologic behaviour of the neonate and puberty may provide conditions under which *Pityrosporum* can colonise infant skin [32].

1.4.3 PITYRIASIS VERSICOLOR

Pityriasis versicolor, like seborrhoeic dermatitis, is a post-pubertal disease which causes cosmetic distress, as the most common features associated with it are the presence of hypo- and hyper-pigmented macules with slight scaling on the chest, upper back, shoulders, upper arms and abdomen [74]. The disease has definitely been determined to be caused by *Malassezia* yeasts and has a propensity to occur in 20-40 year olds and in hot, humid climates. It is a non-inflammatory disease which can be both chronic and superficial.

In pityriasis versicolor, *Malassezia* yeasts convert to mycelial growth [29]. The mycelia hyphae grow downwards into the intercellular spaces between keratinocytes where they feed on non keratinaceous substances and liberate yeast cells which can proliferate and be returned to the skin surface to reinitiate the infectivity process [218]. *Malassezia* filaments are observed in 100% of pityriasis versicolor lesions and in 42% of non lesional skin in affected patients [88,89]. Diagnosis is confirmed by the presence of filaments in skin scrapings/KOH mounts on microscopical examination. In comparison, filaments on normal skin are only observed to a frequency of approximately 6-7% [115]. A study by McGinley *et al* [150] indicated that in lesional pityriasis versicolor skin approximately 3×10^5 mycelia filaments per cm^{-2} were observed in comparison to 1.6×10^5 yeast cells. Non- involved skin areas had populations estimated at 5.8×10^3 mycelial filaments cm^{-2} and 1.9×10^4 yeast

cells cm^{-2}, in comparison to the skin of control subjects with normal skin, where there were approximately 10^3 yeast cells cm^{-2} and few filaments observed.

The clinical symptoms respond well to therapy for cosmetic amelioration by using synthetic detergents combined with various anti-*Malassezia* agents.

1.4.4 *MALASSEZIA* (*PITYROSPORUM*) FOLLICULTIS

Malassezia folliculitis was first described by Weary *et al* in 1969 [229] and established as a clinical and histological entity by Potter *et al* in 1973 [176]. *Malassezia* folliculitis is characterised by discrete, sometimes pruritic papulopustular eruptions localised to the upper part of the trunk, between the shoulder blades and upper arms [176]. Underlying conditions which may predispose to *Malazessia* folliculitis include diabetes mellitus, Cushing's syndrome , renal trauma, bone marrow transplantation, malignancy and immunosuppression [145]. *Malassezia* folliculitis resembles acne in some ways but distinguishing features include the lack of comedones, intense characteristic itch, eruptions mainly on the chest and an older age group than the acne population [85].

Malassezia yeasts as a causative factor in the actiology of folliculitis has been doubted by some investigators[127] but now an eitological role for the yeasts is firmly established [176]. Bäck *et al* [8]showed that on treatment with selenium sulphide shampoo, cure or improvement in cases of *Pityrosporum* folliculitis were

authors proposed yeast overgrowth as a primary factor whilst others proposed that follicular occlusion is the primary factor with yeast overgrowth as a secondary occurrence [108]. What is known is that the disease may either be due to the predisposition of the host in the presence of the organism or the pathogenicity of certain strains of *Malassezia* yeasts being capable of causing follicular lesions. It is also possible that the elaboration of lipolytic enzymes by the yeast within the follicular lumen plays a role in promoting scale formation and disease [108].

1.4.5 ATOPIC DERMATITIS

There is still much debate about the aetiology of atopic dermatitis (AD) which has existed since 1808 [131]. The disease is associated with high levels of allergen-specific immunoglobulin E (IgE) levels, and an immediate-type hypersensitivity reaction to numerous allergens [131]. Genetic factors are also known to underlie the disease [157]. Many different factors may act as allergens in this disease state. These include aero-allergens such as the house-dust mite [14], and pollen [47] as well as allergic and non-allergic food reactions [178]. Micro-organisms have also been shown to play a role in atopic dermatitis, the most notable being *Staphylococcus aureus* [131]. Micro-organisms of the cutaneous flora may also present a stimulus for allergic skin reactions.

The role of *Malassezia* yeasts in atopic dermatitis has been paid much attention over recent years due to clinical and therapeutic evidence. In most patients, sebaceous- rich skin regions will show regional involvement, similar to seborrhoeic dermatitis [131]. Significant improvement of AD on treatment with ketoconazole has been observed [225]. *Malassezia* yeasts may also act as an IgE-binding allergen capable of stimulating cell-mediated immunity in AD to a high frequency showing a positive type-1 reaction in AD, particularly in the head and neck regions [225]. Kieffer *et al* gave evidence for specific IgE antibodies in two-thirds of patients studied with AD as well as positive reactions to epicutaneous tests suggesting the importance of delayed-type hypersensitivity reactions [125]. Increased level of interleukin-4 (IL-4) are also noted [130]. The fact that increased levels of *Malassezia* are not found in atopic dermatitis further indicates that the role of *Malassezia* in AD is as an environmental allergen [32].

In the psoriasis model, further evidence of *Malassezia* as an allergen is given [139]. Lober *et al*, using heat killed sonicated suspensions of *M. ovalis* (*M. furfur*) infected ten psoriatic patients and ten normal patients on the underside of the forearm. Reinitiation of active psoriasis was noted in all test patients who were in a non-active phase of the disease. A response to *M. ovalis* was also observed in two out of ten control patients who had a history of seborrhoeic dermatitis, thus providing a further link between the two disease states [139].

Several IgE binding components have been observed in *P.orbiculare* (*Malazzesia)* extracts [245]. Zagari *et al* [245] raised several monoclonal antibodies to *P.orbiculare* in an attempt to characterise potential surface antigens. Two IgE

monoclonal antibodies showed distinct bands of molecular masses 67kDd and 37 kDa. Both components were observed to be IgE binding components [245]. In 1997, Zagari *et al* [246] investigated the subcellular locations of these proteins within the genus *Pityrosporum* by use of confocal laser scanning microscopy and flow cytometry. Ninety five percent of the *P. orbiculare* (*P. ovale*) cells from a four day culture showed cell surface binding of an anti-37kDa monoclonal antibody (MoAb) and 88% of cells to an anti 67kDa MoAb. It was also concluded that all the members of the genus *Pityrosporum* express the 37 kDa and 67 kDa major allergens on the cell surface, which were not detected in *Candida* species and *Saccharomyces cerevisiae* controls [246]. The 37 kDa protein has now been named Malf1 and the complete cDNA encoding this allergic protein has been established. Malf1 has now been expressed as a maltose-binding fusion protein in *Escherichia* and has been shown to react with specific monoclonal antibody and with IgE from atopic dermatitis sufferers [189]. The work from this team may have great implications on our current knowledge of *Malassezia*-related infections. It may be that the role of *Malassezia* yeast cells in dandruff and seborrhoeic dermatitis may be as an allergen rather than as a conventional pathogen. If this is the case, then for various *Malassezia* disease states, severity may be limited by the use of relatively non-allergic peptides that may down regulate IgE response and relieve the disease state [246].

1.4.6 OTHER DISEASES ASSOCIATED WITH *MALASSEZIA* YEASTS

1.4.6.1.CATHETER-ASSOCIATED SEPSIS

This iatrogenic infection usually occurs in low birth weight infants and occurs infrequently as a complication of use of central venous catheters for long term venous access with administration of hyperalimentation fluids containing lipid emulsions [58,177]. Colonisation of the catheter provides the organisms with access into the bloodstream causing sepsis [145]. Barber *et al* suggests that this type of fungemia occurs in immunocompromised patients with central venous catheters whether or not a lipid treatment is being given [11]. Evidence of such cases is given elsewhere [58]. It is possible that contamination of catheters at time of placement occurs [145], though it is more likely that the organisms are present from colonised skin along the subcutaneous catheter tract and outer catheter wall [145]. Until the main source of contamination is established, further study of catheter colonisation is needed so that the pathogenesis of *Malassezia* catheter-related sepsis can be established.

1.5 IMMUNOLOGY

In the pityriasis versicolor model, it appears that colonisation with *Malassezia* yeasts is associated with the development of antibodies; however, there is a connection between the duration and magnitude of colonisation as well as the presence of actual infection, and elevated antibody titres, [145]. Some evidence of this phenomenon came from the work of Faergemann *et al* [73]. Using indirect immunofluorescence to determine the antibody response to *Malassezia*, Faegermann observed that no differences in antibody titre arose between patients and adult controls. This work was supported by evidence obtained from a study by Furukawa *et al* in 1981 [87]. In two conflicting studies, a higher antibody response has been found in pityriasis versicolor patients compared to age and sex-matched controls [55,241]. Essentially, significant differences in antibody titres to *M. furfur* patients with autonomous disease and controls are apparent when a sensitive antibody detection technique is used. Enzyme immunoassay (EIA) techniques have been used to this end. EIA studies have shown low antibody titres in six month old children [145] which indicated that EIA may be sensitive enough to detect differences between colonised and infected individuals. From these studies it has been shown, however, that high antibody levels to *Malassezia* yeasts are observed in pityriasis versicolor which may suggest that the antibody response is non-protective, but it is not thought that cell-mediated immunodeficiencies are involved in pityriasis versicolor as this group of patients show no increase in susceptibility to other infections. Relatively few studies have investigated the interaction between *M. furfur* and specific cell-mediated immune defence.

In the majority of individuals, *Malassezia* yeasts do not cause overt disease. It appears that in some *Malassezia*-related infections, host immunocompromisation may be involved in the pathogenicity of the disease, *Malassezia* yeasts beginning to act as opportunistic pathogens.

Unlike the yeast to mycelial transformation in pityriasis versicolor, the changes causing the conversion from commensal to pathogen in other *Malassezia*-related infections are unknown.

Cutaneous diseases caused by *Malassezia* yeasts are non-invasive. *Malassezia* does not penetrate the epidermis in seborrhoeic dermatitis and penetrates no further than the dermis in cases of pityriasis versicolor. In folliculitis, although the yeast may reside deep within the pilosebaceous follicle, it is essentially separated from the host's dermal tissue by the follicle wall. While the cutaneous manifestations of *Malassezia*-related diseases remain superficial, it is unknown whether deeper penetration is inhibited by nutritional requirements or host defence mechanisms.

In normal individuals, antibodies specific to *Malassezia* yeasts have been demonstrated. Cunningham *et al* [53] demonstrated measurable levels of IgG and IgM in two and three year olds. These antibody levels were not observed to change into adulthood. IgM titres were the only titres apparently affected by age, where a decrease in levels were observed in elderly individuals compared to younger subjects [211].

In pityriasis versicolor, a moderate accumulation of predominantly CD4 positive T-helper cells have been observed in the dermis and epidermis although this cellular infiltrate is normal in comparison to the fungal load [105]. However, Wu and Chen [240] assessed the transformation response of lymphocytes from 31 patients with pityriasis versicolor and 30 normal volunteers to *M. furfur* and reported a higher lymphocyte transformation index in the patient group.

In seborrhoeic dermatitis, there is evidence of mononuclear cell infiltrates in histological sections of lesional skin. In 1991, Bergbrant *et al* [21]observed heavy dermal infiltrates in lesional biopsies of seborrhoeic dermatitis, and no differences in three normal biopsies when lesional and nonlesional skin of seborrhoeic dermatitis patients was examined. It was observed that the majority of infiltrating cells were CD4 + T- helper cells. Similar observations are in line with the lack of visible inflammation in pityriasis versicolor compared to the erythema associated with *M. furfur* folliculitis.

Ashbee *et al* [7], showed by lymphocyte transformation assay and leukocyte migration inhibitions assay that there was an absence of cell mediated immunodeficiency to *M. furfur* in pityriasis versicolor or seborrhoeic dermatitis patients. They postulated that the T-cell response observed may indicate that T-lymphocytes might be involved in the pathogenesis of these diseases. In a similar study on humoral immunity to *M. furfur*, no difference in humoral response to *M. furfur* between patients of seborrhoeic dermatitis, pityriasis versicolor and normals was observed, and it was thought that humoral immune response is not related to *Malassezia* pathogenesis of infection, but merely to *Malassezia* carriage [6].

However, evidence is now emerging that *Malassezia* may act as an allergen in some disease states [246], so it may be that humoral response is important in *Malassezia*-related infections, which may also help clear up the historical dilemma of why some people show colonisation by *Malassezia*, with no infection, whilst other may develop *Malassezia* -associated disease.

1.6 TREATMENT OF MALASSEZIA RELATED INFECTIONS

Treatment of *Malassezia*-related skin infections is normally prolonged and there may be a propensity for frequent relapse. Topical treatments include the use of 2-5% selenium sulphide, ketoconazole or miconazole creams in the treatment of pityriasis versicolor. The condition is also known to respond well to oral ketoconazole. Selenium disulphide is fungitoxic and is thought to exert its affect by irreversibly changing the free sulphydryl groups found in the cell wall into polysulphide bonds, preventing cell division [33]. However, topical selenium sulphide treatment in pityriasis versicolor has been found to have a highly frequent relapse rate. Systemic ketoconazole treatment is much more effective. In one study, over four weeks , oral ketoconazole (200mg) was effective in producing a cure in 97% of a pityriasis versicolor patient population. Only 36% of this population showed relapse after 12 months post treatment [198]. Although no adverse side effects were observed over a four week duration, long term therapy with ketoconazole is inadvisable for continuous infections due to the risk of hepatotoxicity and interference with testosterone metabolism [37].

Oral itraconazole therapy is regarded as a safer treatment for pityriasis versicolor than ketoconazole treatment. A study by Delcleuse *et.al.*[60] showed that 200 mg itraconazole daily for five days was an effective treatment for pityriasis versicolor. Abnormal liver function has been found in 4% of patients receiving oral itraconazole therapy for long term systemic fungal disease although 50% of these patients has a previous history of liver weakness [42]. Patients show a better tolerance to itraconazole than selenium sulphide [60] and there is now much evidence supporting a less toxic mode of action of itraconazole than ketoconazole [61]. The advent of an oral treatment with low toxicity has therefore prompted a move away from the topical treatments which are messy for application with a lower efficiency than oral actives [115].

Scalp seborrhoeic dermatitis and dandruff have both been observed to be cleared effectively with 2% ketoconazole shampoo or 2.5% selenium sulphide shampoo [37,56,170]. Two percent ketoconazole shampoo has been shown to also prevent relapse when used as a weekly prophylactic treatment [170] and is also better tolerated than selenium sulphide shampoo [56]. Topical 2% ketoconazole cream treatment has also proven to be effective in treatment of seborrhoeic dermatitis non-scalp lesions [209]. In comparison to 1% hydrocortisone cream, 2% ketoconazole cream has less side effects and proves to be more effective [120].

Zinc pyrithione may also be used as a topical treatment. This active is also classified as an antikeratolytic but causes a reduction in *Malassezia* numbers by effects upon membrane transport and macromolecular synthesis [152]. Nystatin has also been

used in anti-dandruff formulations, but *Malassezia* can become nystatin- resistant and cause reinfection [152].

Ointments containing 8% lithium succinate and 0.05% zinc sulphate are also effective anti-seborrhoeic dermatitis agents. The precise mode of action if lithium succinate in seborrhoeic dermatitis is unknown [69] although lithium is known to have a growth inhibitory effect on small colony strains of *Malassezia* [136]. Lithium is also known to block the release of free fatty acids in tissues, so therefore may play a role in reducing the *Malassezia* growth substrate thus having a direct and indirect effect on growth [69]. Oral ketoconazole can provide effective systemic treatment for seborrhoeic dermatitis, but as with pityriasis versicolor, prolonged therapy is inadvisable [115].

The mode of action of ketoconazole and other imidazoles is perhaps better understood that the other anti-*Malassezia* treatments. Itraconazole is a triazole derivative and ketoconazole is an imidazole derivative of the azole antifungal group. These azole derivatives both belong to the class of 14α-demethylase inhibitors. They are known to inhibit the cytochrome P450- dependent lanosterol 14-demethylase activity of fungi, an integral step in the formation of ergosterol. Ergosterol is the main cell membrane sterol in fungi, necessary for growth and proliferation and plays architectural and functional roles. Disruption of ergosterol biosynthesis leads to growth inhibition or cell death.

Ketoconazole and itraconazole exert their activity in the fungal endoplasmic reticulum. Inhibition of the cytochrome P-450 isoenzyme (P-450, 140 DM) results in

the inhibition of P-450 catalysed reactions [244]. This causes an accumulation of 14-methyl sterols such as lanosterol causing membrane instability and compromising cell growth [152]. As more becomes understood about processes involved in *Malassezia* growth and infection, more antifungal novel targets may be elucidated and more choice of effective anti-*Malassezia* agents will become available.

2. Chapter 2 : Yeast-Hyphal Transition in *Malassezia* Species.

2.1 SUMMARY

Malassezia yeasts are lipophilic yeasts capable of existing in the yeast form and in some cases in the hyphal form. The hyphal form is commonly observed in cases of pityriasis versicolor.

An investigation into factors affecting the yeast-hyphal transformation in *Malassezia* yeasts was undertaken, including a review of existing methods of filament production [63,73,76,159]. Scanning electron microscopy (SEM) was used to make a detailed study of hyphal length and structure under various conditions. Squalene and cholesterol in the presence of stripped stratum corneum (ssc) were found to induce filament production in three *Malassezia* species, under the classification of Guého *et al* [97]. A role for magnesium in *Malassezia* dimorphism is also proposed. This investigation is discussed in relation to existing methods of hyphal induction in *Malassezia* species, and has shown that many factors are involved in the yeast-hyphal transition for *Malassezia* species which are both species dependent and environmentally dependent.

2.2 INTRODUCTION

In various mycoses, the capacity to form hyphae may play a major role in pathogenicity, and in many cases this transition is associated with a change from saprophyte to pathogen.

Hyphae are comprised of multiple fungal cell units which are partitioned by septa. Yeast cells may produce hyphae and hyphae may also form as branches of existing hyphae. In most cases, hyphal production is a consequence of new cellular material growing in an elongated manner known as a germ tube and expanding by apical extension. Septa are formed when mitotic cell division occurs and do not disrupt the rate of expansion.

It is well documented that fungi such as the dermatophyte fungi and yeast-like fungi such as *Candida albicans*, have the capacity to grow in a filamentous manner. In other pathogenic genera, filaments will be formed *in vitro* at room temperature, although *in vivo*, the yeast form is found to cause disease. "Dimorphism" is usually the term used to describe this variation in growth forms, although in many cases it is a misnomer as various fungi such as *Malassezia* and *Candida* species display pleomorphic growth patterns [165].

The capacity to form hyphae is displayed by many pathogenic yeasts including *C. albicans ,and Saccharomyces cerevisiae* [92]. Filament formation by *Malassezia* species is also well documented in cases of pityriasis versicolor [121]. Early investigators observed the hyphal phase of *M. furfur* from lesional skin samples of patients with pityriasis versicolor [70, 210]. These filaments were named by Robin in 1853.[191]. Tropical forms of pityriasis versicolor were thought of as a separate disease (pityriasis flava) from pityriasis versicolor in temperate zones [38,39]. The filaments were classed in the genus *Microsporon* [191] and the disease was proposed to be caused by a fungus termed *Malassezia tropica* [40]. The confusion in classification arose as some investigators could not provide evidence of a connection between the filaments observed in pityriasis versicolor lesions and *Malassezia* species. Some early investigators provided a link between the yeast and mycelial phases of *Malassezia* through the consistent observation of either the yeast phase or the hyphal phase being associated with chronic skin scaling disorders [128, 195]. Panja [167] was the first investigator to classify the yeast and mycelial forms into a single genus in 1927, and it is generally accepted that he was the first investigator to successfully subculture *M. furfur* [167]. At this time, the concept of a transition between yeast growth and filamentous growth in various fungal systems was not accepted by many investigators and the yeast was classified in the genus *Pityrosporum*, described as oval or bottleshaped cells with no mycelium [140]. However, evidence accumulated linking the yeast and the mycelial fungus observed in pityriasis versicolor, and the organism was consequently reclassified in the second edition of "The yeasts. A taxonomic study"[141] in the genus *Pityrosporum* as "Globose, oblong ellipsoidal to cylindrical shaped cells with hyphae developing rarely and sparingly in artificial culture". At this stage, all evidence of a yeast-

mycelial transition was provided from *in vivo* observations and had not been demonstrated *in vitro*. Conclusive *in vitro* evidence was not provided until 1977 when Dorn *et al* [63]and Nazarro-Porro *et al* [162] resolved the debate of the morphological transition in *Pityrosporum* species.

The yeast-hyphal transition cannot be thought of as under the influence of or being the consequence of one environmental parameter alone. In some yeast-like fungi, the transition to hyphal growth from yeast growth may be selected for by various parameters although many of these have been shown to be nonessential for filamentous growth [166]. In contrast, in other dimorphic fungi, a single environmental trigger can be shown to be essential for the yeast-mycelium transition. For example, temperature induces this transition in *Histoplasma capsulatum* [92] whilst in *Mucor* species, the filamentous form tends to grow under anaerobic conditions [92].

The equilibrium between yeast and hyphal growth and the factors affecting it are probably most well documented in *C. albicans*. Many different switches may be involved in the yeast-hyphal transformation, suggesting that several independently-regulated cell-signalling systems are involved [92]. For example, N-acetylglucosamine and serum are known to induce the yeast-hyphal transition in *C. albicans* and operate independently [166]. Other factors which select for hyphal growth include elevated temperature, neutral pH and relatively nutrient poor growth [166]. Second messenger systems such as those based upon cAMP [43], Ca^{2}+, calmodulin- and inositol-phosphates, and intracellular pH have all been implicated in the yeast-hyphal transition in *C. albicans* [110]. Exogenous addition of second

messengers, their precursors or analogues may induce hyphal production in *C. albicans* [93]. Further evidence is given when it is shown *in vitro* that the addition of second-messenger inhibitors may also retard growth of *C. albicans* in the hyphal growth phase. It is also proposed that the expression of certain genes under the control of a second messenger may act in the capacity of a "master switch" which controls morphological behaviour. This hypothesis is, however, still under debate [166].

Few factors have been elucidated in having an involvement in the yeast-hyphal transition in *Malassezia* yeasts, in comparison to the *C. albicans* model. Burke was the first investigator to produce hyphal filaments in cultures of *M. furfur* [35]. Conclusive proof of this phenomenon *in vitro* was given by the work of two separate groups in 1977. Dorn and Roenhert[62] reported in 1977 that globose cells of *P. orbiculare* readily converted to hyphal elements in a defined medium. The culture consisted of 0.05M glycine in 0.03-0.06M ammonium phosphate buffer (pH 5.6), salts, glucose and Tween 80. Of the strains tested, 78-85% produced hyphae from up to 8.5% of yeast cells at 29°C. This transition was not observed in any *P. ovale* strains tested. In the same year, Nazarro-Porro *et al* [162] studied the effect of complex lipids such as cholesterol and cholesterol esters on the *in vitro* growth and induction of hyphae in *M. furfur* isolates. They successfully induced hyphae on yeast morphology agar (YMA) containing cholesterol, cholesterylstearate and glycerolmonostearate [162]. They indicated that the mycelial type of growth in culture closely resembled that of *M. furfur* in tissue, and concluded therefore that the mycelial form of the organism is thought to represent the invasive, pathogenic form [162].

An *in vitro* stratum corneum model as a method of induction of hyphae in *Pityrosporum ovale (orbiculare)* was proposed by Faergemann in 1989 [76]. *P. ovale* was cultured directly on sterile pieces of stratum corneum tissue, obtained from cadaver skin, placed directly upon glucose neopeptone-yeast extract agar and incubated at 37°C in a microaerophilic environment. Filament production was observed after 6 days incubation under these conditions. This transition was also observed to be lost on subculture. Faergemann also proposed that the age of the inoculating culture was also a factor in the yeast-hyphal transition *in vitro*. Strain-dependent factors were also observed. Other work by Faergemann and co-workers gives evidence of a potential role for CO_2 in the yeast-hyphal transition in *Pityrosporum* species [75,76].

In the previous literature, strain variation has been involved in the yeast-hyphal transition. In some cases, more strains of a single *Malassezia* species were observed to be unable to form filaments in comparison to the number of strains showing a filamentous capacity [76]. These discrepancies may be due to the general confusion which has surrounded the classification of these organisms throughout the years of investigation. In 1996 a new taxonomical classification was proposed, encompassing seven distinct species [97]. It may become apparent in future that only some species have the capacity to form hyphae and may aid further elucidation of pathogenic mechanisms associated with *Malassezia*-related infections. It is for such reasons that this study was under taken.

2.3 AIM OF STUDY

This investigation was undertaken to elucidate factors involved in induction of the yeast-hyphal transition in *Malassezia* yeasts and to confirm the reports of previous workers. A further aim was to elucidate a link between the yeast-hyphal transition and species classification according to Gueho *et al* [97]. By carrying out this study it was hoped to broaden our understanding of the yeast-hyphal transition and its role in pathogenicity of *Malassezia*-related infections.

2.4 MATERIALS AND METHODS

2.4.1 Isolates

An *M. furfur* strain designated D82412, was obtained from a case of pityriasis versicolor and an *M. furfur* strain designated as "Hook" was isolated on Dixon's agar from a scalp psoriasis study at the Western Infirmary Glasgow, UK. Isolates GM110 (*M. slooffiae*), GM340 (*M. sympodialis*) and L251 (*M. furfur*) were kindly provided by Dr G Midgley, St. John's Institute of Dermatology, St. Thomas Hospital, London, UK. Isolate 0333.1 (*M. furfur*) was kindly provided by Unilever Research , Wirral, UK.

All isolates were grown for 24h in modified Dixon's broth at 29°C. Five ml aliquots were then centrifuged and washed three times in Dulbecco's Phosphate Buffered Saline (PBS) before agitation with glass beads to achieve a single cell suspension. Yeast cell counts were then carried out with a haemocytometer and subsequently diluted to achieve stock suspensions of approximately $1x10^7$ yeast cells ml^{-1}.

2.4.2 Media components

Squalene, cholesterol, cholesteryl monostearate, glycine, potassium nitrate, magnesium sulphate, sodium chloride, glucose cyclohexamide, chloramphenicol, and ferrous sulphate were obtained from Sigma chemicals UK. Tween 20 and Tween 80 were obtained from BDH and Tween 40 was obtained from Merck Schuchardt, Hohenbrunn, Germany.

2.4.3 Stripped stratum corneum

D-squame discs (CuDerm, Texas) were applied to the skin of volunteers, the procedure being repeated five times until the discs were no longer sticky. This procedure removed the outermost layers of the stratum corneum. In the experiments included in this study, stripped stratum corneum samples were taken from a site at the back of the subject's necks. Preliminary studies indicated that there were no

significant differences in stratum corneum sites taken different donors. In some cases, the samples were saturated in PBS-solution containing either cholesterol, cholesterol and oleic acid, or squalene. After this process was carried out, samples were carefully removed using forceps and inoculated with 20µL of 1x 10^6 yeast cells of various *Malassezia* isolates and were placed on glass slides resting on glass rods in a petri dish containing five ml distilled H_2O to provide a humidified environment. All glassware had been surface sterilised. The samples were then incubated at 37°C for up to five days. After incubation, samples were fixed and processed for scanning electron microscopy (SEM).

2.4.4 Cultural studies

A reevaluation of the method used by Dorn and Roenhert in 1977 [63] to induce filamentation in *P. orbiculare* was undertaken with various species. This medium consisted of (gl^{-1}) : potassium nitrate,1.0; magnesium sulphate, 0.13; sodium chloride, 1.3; glycine, 3.75; glucose ,13; cycloheximide, 0.5; chloramphenicol, 0.05; ferrous sulphate, 0.6 mgl^{-1}, Tween 80, $50ml^{-1}$ in 0.06M ammonium phosphate buffer (pH 5.6). In a series of experiments, each individual component was removed systematically from 200ml aliquots of broth and *Malassezia* isolates examined for the yeast-hyphal transition after 5d incubation at 29°C in an orbital incubator. (This work was not carried out at 37°C because the original authors used this lower temperatures. However, no differences in capacity to form filaments at 29 °C or 37 °C were observed in preliminary experiments). When

conditions of modified Dorn's medium were found to prevent filamentous growth, each component was then individually reintroduced to the remaining media components to examine whether any particular component stimulated filamentation. Extent of filamentation was calculated by counting 10 fields of 100 cells and calculating what percentage of filaments was present over these fields. Various Tween (T) supplements were also substituted into this medium to observe any effect they had on hyphal stimulation.

2.4.5 Lipid supplemented agar and broth cultures

Isosensitest broth and diagnostic sensitivity agar (DSA) obtained from Oxoid, Basingstoke, UK, were used as the basal media. In each case, the medium was supplemented with either 8% squalene or a suspension of cholesterol:cholesteryl stearate: glycerol monostearate at a ratio of 2.0:1.5:2.0 as outlined by Nazarro-Porro *et al* [162]. Incubation was carried out at either 29°C or 37°C for five days after which samples were processed for light- or electron microscopy.

2.4.6 Scanning electron microscopy (SEM).

After incubation, stripped stratum corneum samples were fixed in 2.5% gluteraldehyde in 1.0M Sorrenson's buffer (see appendix), pH 7.4 for 1h. After washing in buffer for 45min the adhesive discs were postfixed in 1% osmium

tetroxide and rinsed again in buffer followed by successive dehydration steps in graded ethanol solutions (25-100%), substituted with liquid carbon dioxide, dried in a critical point dryer, mounted on 10mm aluminium stubs (Jeol, UK), sputter coated with gold and examined in a JSM 6400 scanning electron microscope.

2.4.7 Statistical analyses

Where replicate experiments had been carried out to verify the percentage of filamenting cells in each experiment, the standard error of the mean (sem) and the standard deviation (SD(n-1)) was calculated These have been represented graphically in the form of error bars.

2.5 RESULTS

2.5.1 Stripped stratum corneum

In the presence of either 8% squalene or 1.5% cholesterol, filamentation was observed with various *Malassezia* isolates up to 76% in the presence of squalene and

16% in the presence of cholesterol. The presence of oleic acid did not increase the percentage filamentation when added to cholesterol samples (Figure 2.1). In each case the filaments observed were approximately 30-65μm in length with an irregular shape, compared to the short filaments normally isolated from pityriasis versicolor lesions (Figure 2.2 to Figure 2.6). Filaments could be observed extending from various points on the parent cell surface. Branching was also observed (Figure2.7., Figure 2.8). The phenomenon of phialospore extrusion was observed under all conditions examined (Figure 2.9 to Figure 2.11). Filaments grew in clusters and extended along corneocyte surfaces. Invasion into the stratum corneum was not observed (Figure 2.12, Figure 2.13). Control samples which did not contain a lipid supplement did not stimulate filamentous growth in any of the isolates examined (Figure 2.14).

In some cases a coating was observed to form over clumps of yeast cells (Figure 2.15, Figure 2.16). Plasmodesmas were also apparent which joined adjacent cells or formed a bond with the stratum corneum tissue, similar to those observed in catheter colonisation [145]. These structures were not observed under light microscopy, but were observed under most of the conditions examined by SEM. (Figure 2.17).

2.5.2 Dorn's Medium

When Dorn's medium was examined a variety of isolates representing the species *M. furfur, M. slooffae* and *M. sympodialis* formed filaments at 29°C but not at 37°C [not shown].

When the basal medium was supplemented with Tween (T) 20, Tween 40 or Tween 80 and isolates incubated at 29°C for 5 days both the T80 and T20-supplemented culture showed filamentous growth for all isolates examined whilst T40-supplemented culture supported growth in the yeast phase (Figure 2.18). Removal of individual media components revealed that filamentation did not occur on removal of ferrous sulphate, magnesium sulphate or glycine (Figure 2.19). When each of these components was resubstituted individually to the remaining media constituents, filamentation in *M. furfur* only occur upon reintroduction of magnesium sulphate, though to a much lesser extent than in the original medium (not shown).Under the conditions examined, the filaments produced bore greater resemblance to those observed on the stripped stratum corneum samples. Other species examined failed to produce filaments.

2.5.3 Lipid supplemented media

Filamentous growth was not observed in any cholesterol or cholesterol-supplemented media. Various components could be seen to precipitate from the media which hindered growth. In liquid culture, squalene as a sole lipid supplement did not stimulate growth in the yeast or hyphal phase. However, in agar medium,

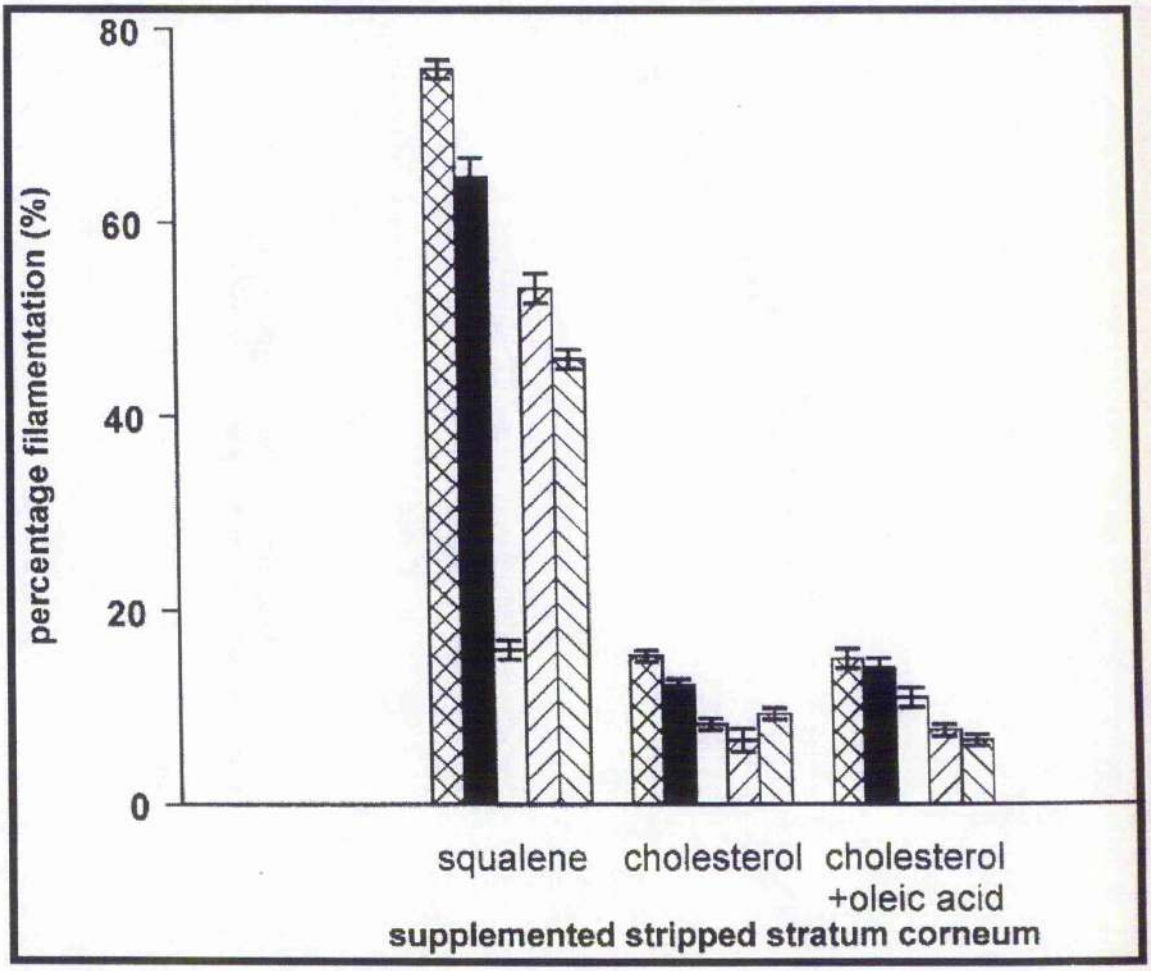

Figure 2.1. Growth of *M. furfur* isolates in the presence of squalene, cholesterol or cholesterol and oleic acid. Isolates were observed to produce filaments up to 76% in the presence of squalene and up to 16 % in the presence of cholesterol. Results are averaged over a series of three experiments. Hook ⊠ GM110 □ GM340 ▨ L251 ▧ D82412 ■

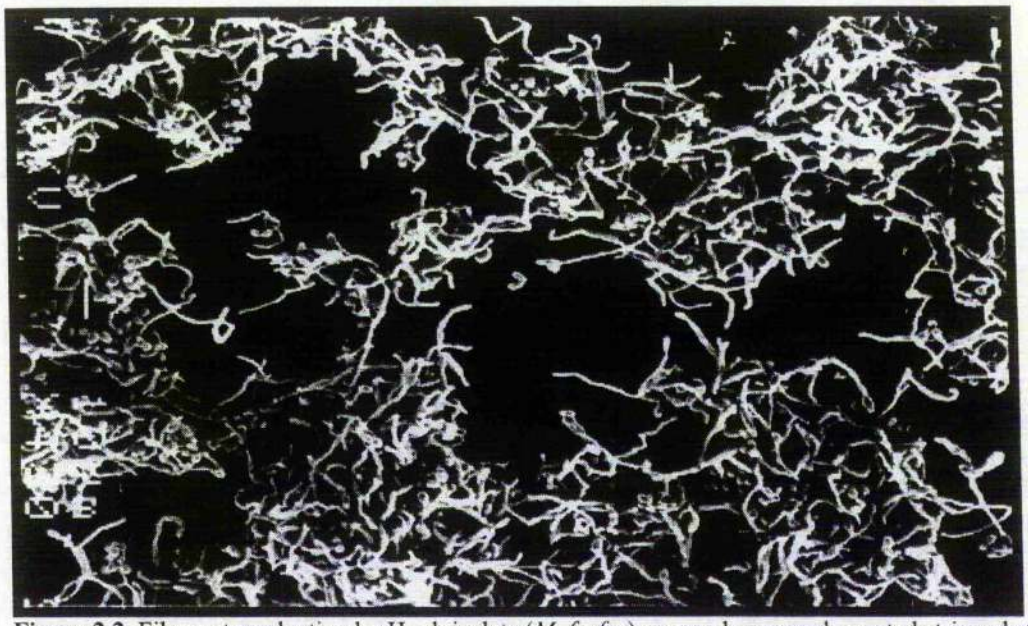

Figure 2.2. Filament production by Hook isolate (*M. furfur*) on squalene-supplemented stripped stratum corneum sample. A very high percentage of filamentation was observed with filaments being long, branched and irregular (X1,000)

Figure 2.3. Filament production by *M. furfur* on 1.5% cholesterol-supplemented stripped stratum corneum sample. Filament production was observed to a lower frequency than in the squalene-supplemented samples. Branched and irregular filaments were observed (x2000).

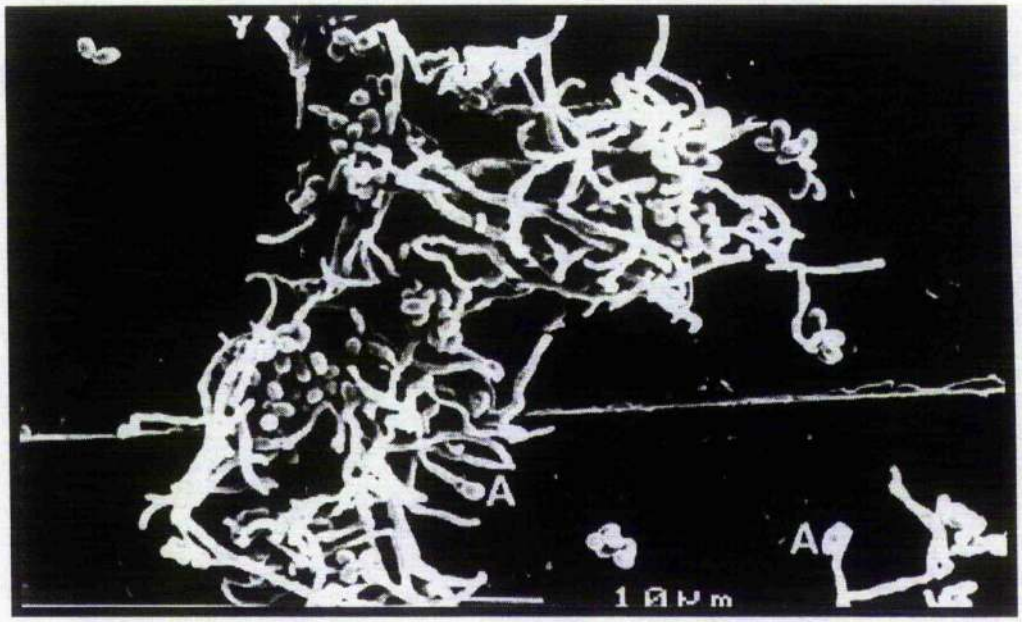

Figure 2.4. Filament production by *M. furfur* on 8.0% squalene-supplemented stripped stratum corneum sample. Tangles of irregular-shaped filaments are observed in association with clumps of yeast cells. Filaments are long and phialospore extrusion is observed (X1,500).

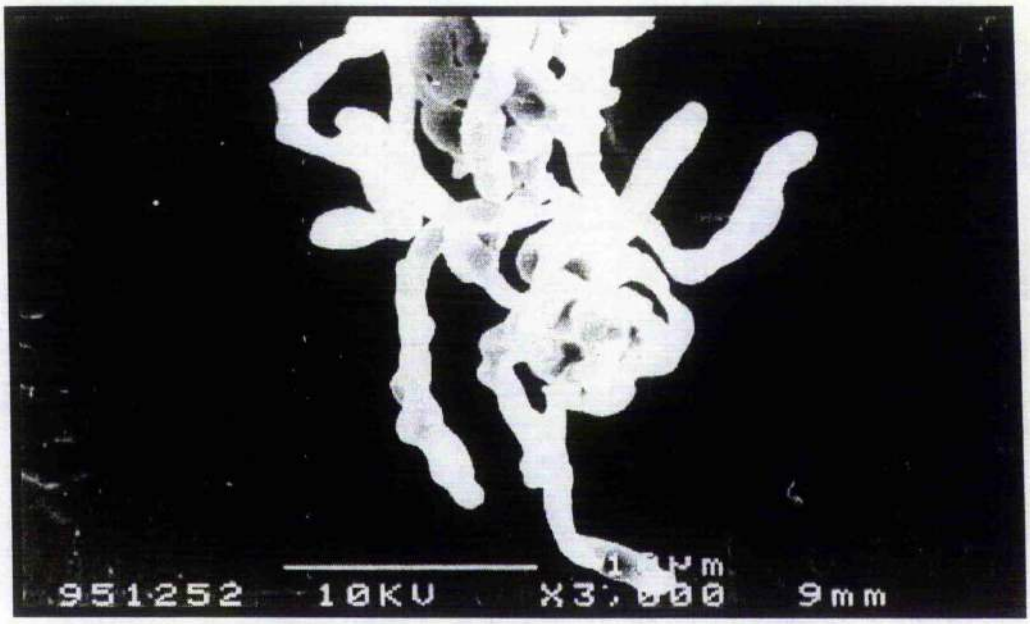

Figure 2.5. Filament production by *M. furfur* on 1.5% cholesterol-supplemented stripped stratum corneum sample. Tangles of irregular filaments in association with groups of yeast cells are observed. Branch formation is observed (X3,000).

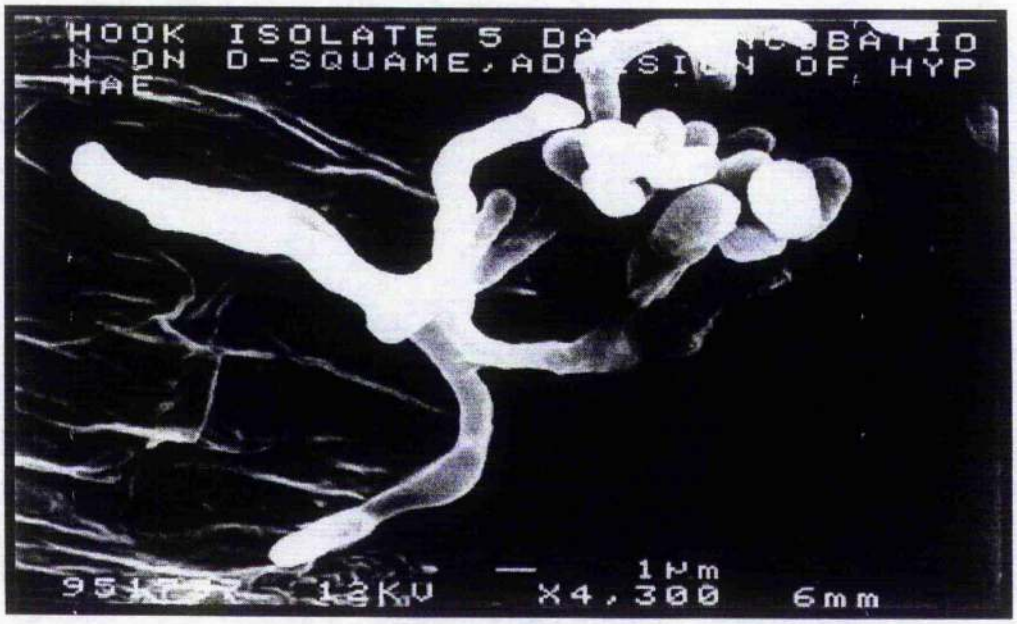

Figure 2.6. Filament production by *M. furfur* on 1.5% cholesterol and oleic acid-supplemented stripped stratum corneum sample. Branched irregular filaments in association with clumps of yeast cells and corneocyte surfaces (X4,300).

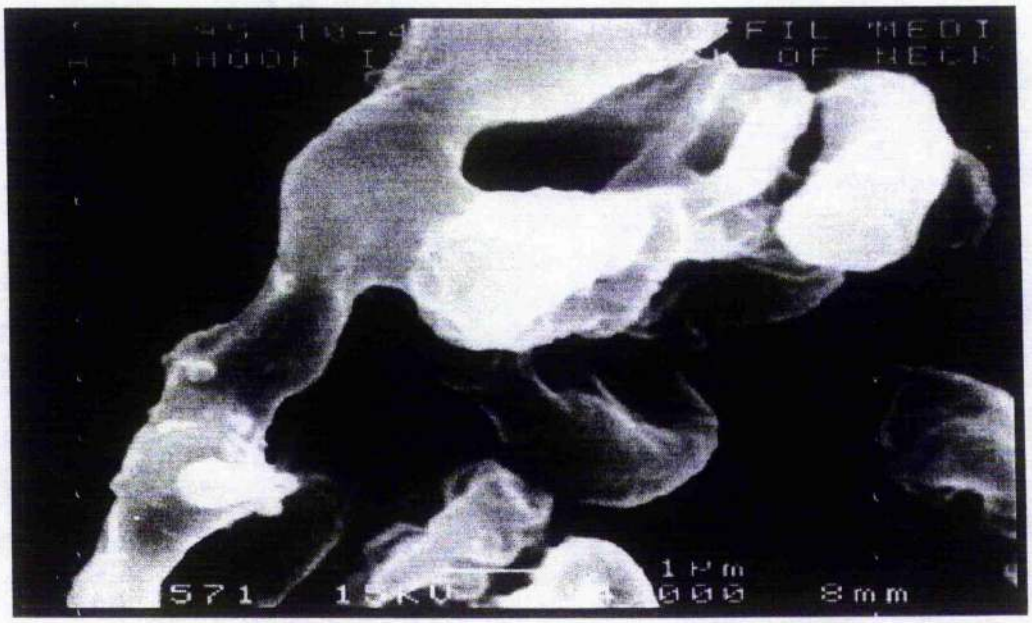

Figure 2.7. Branching filaments of *M. furfur* observed on squalene-supplemented stripped stratum corneum sample (X14,000).

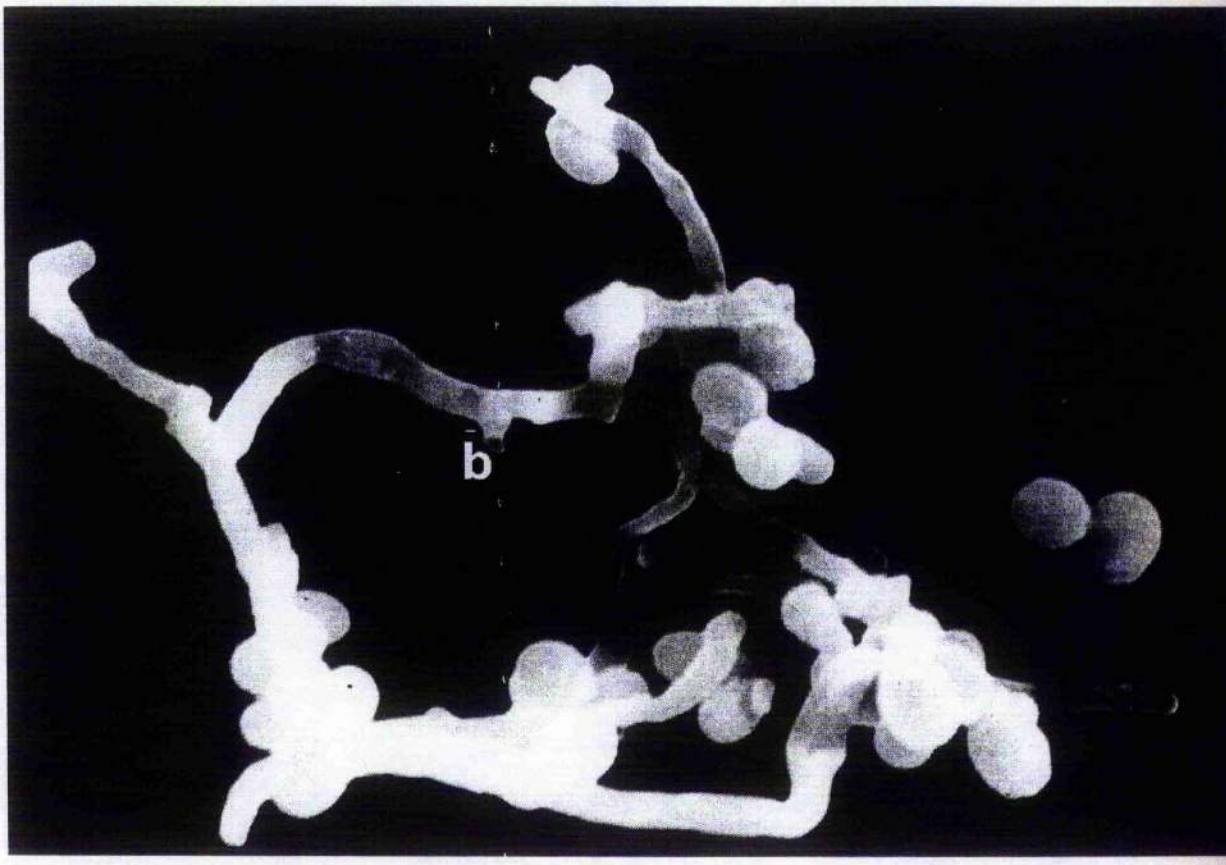

Figure 2.8. Branching filaments observed in Hook isolate on stripped stratum corneum samples supplemented with 8% squalene (x3,000).

Figure 2.9. Phialospore extrusion by *M. furfur* on 8% squalene-supplemented stripped stratum corneum samples. Phialide cells were extruded at on end (p) whilst the hyphae extended at the other end (arrow).

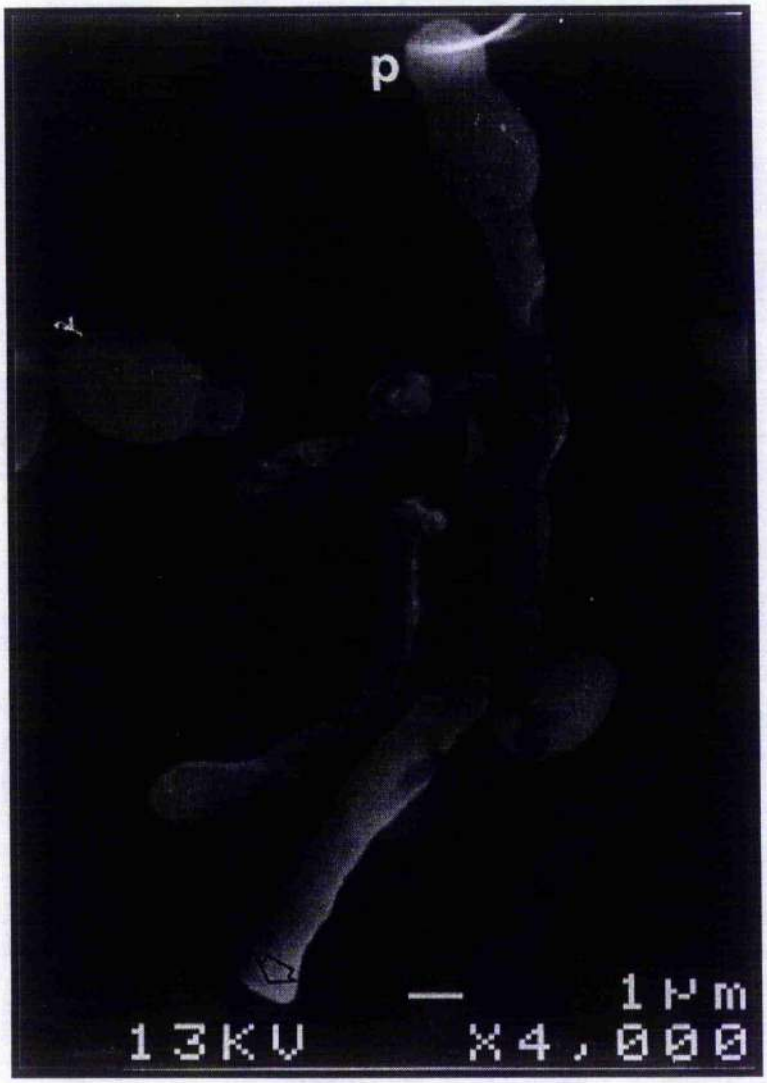

Figure 2.9. Phialospore extrusion by *M. furfur* on 8% squalene-supplemented stripped stratum corneum samples. Phialide cells were extruded at on end (p) whilst the hyphae extended at the other end (arrow).

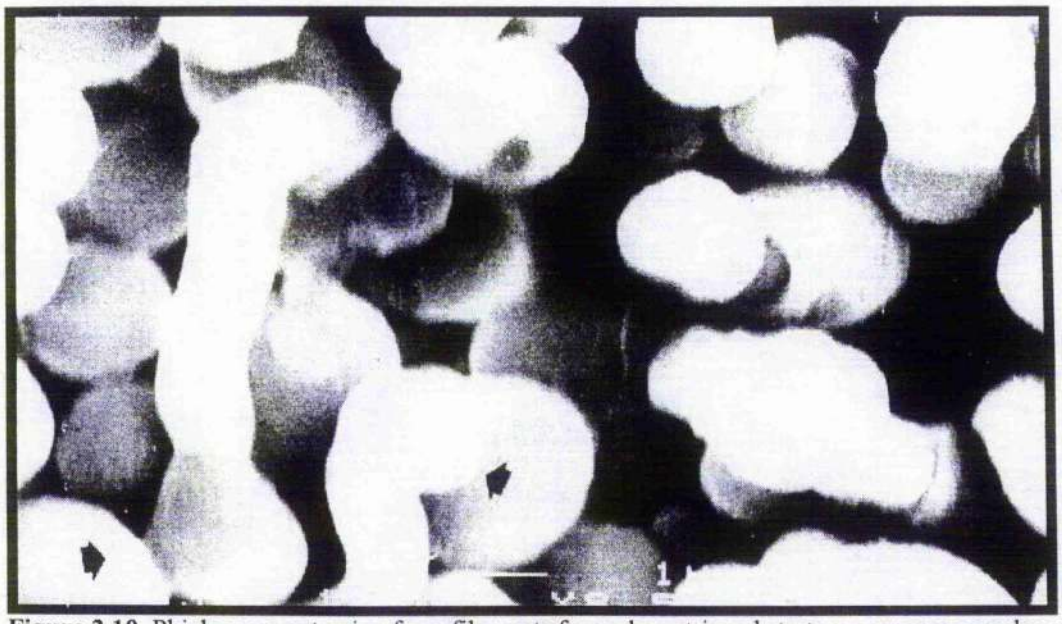

Figure 2.10. Phialospore extrusion from filaments formed on stripped stratum corneum samples supplemented with 1.5% cholesterol. Phialospore extrusion (arrows) could be observed in the presence of budding yeast cells (X8,500).

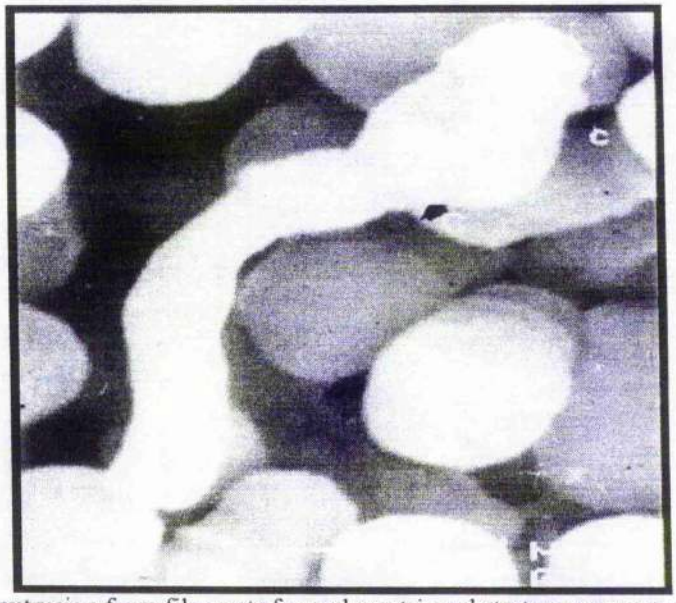

Figure 2.11. Phialospore extrusion from filaments formed on stripped stratum corneum samples supplemented with 8% squalene. A distinctive collarette (c) where the phialospore is extruded. The parent cell site from which the filament extends has a constriction (C) in comparison to the broad collar where the phialide buds off.

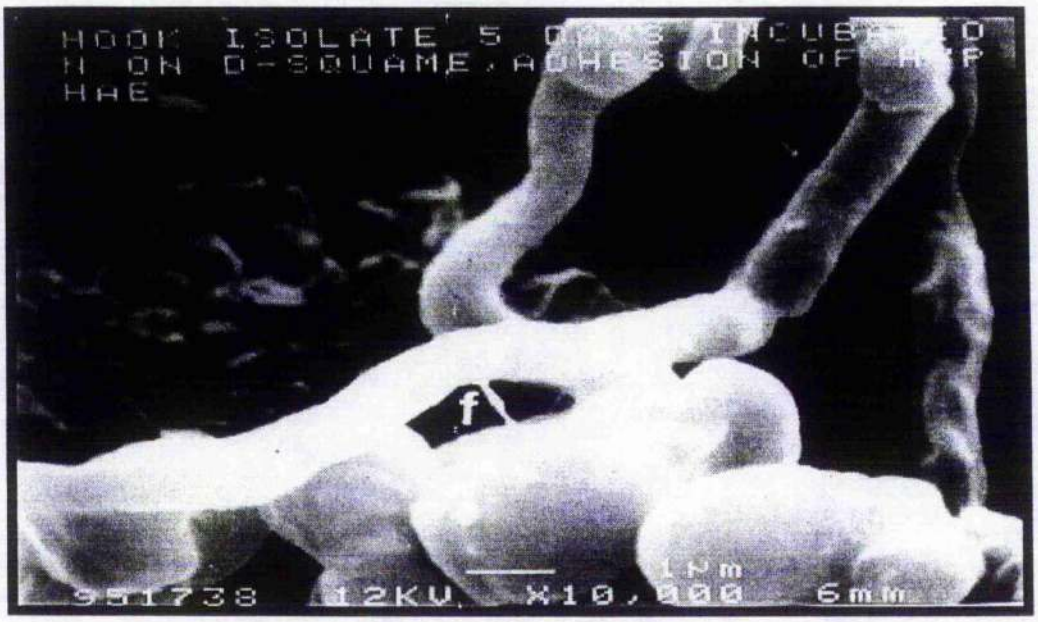

Figure 2.12. Filaments in association with clumps of yeast cells. Constricted irregular-shaped filaments observed with fibrin-like structures observed to attach between the hyphal wall and yeast cell wall (X10,000).

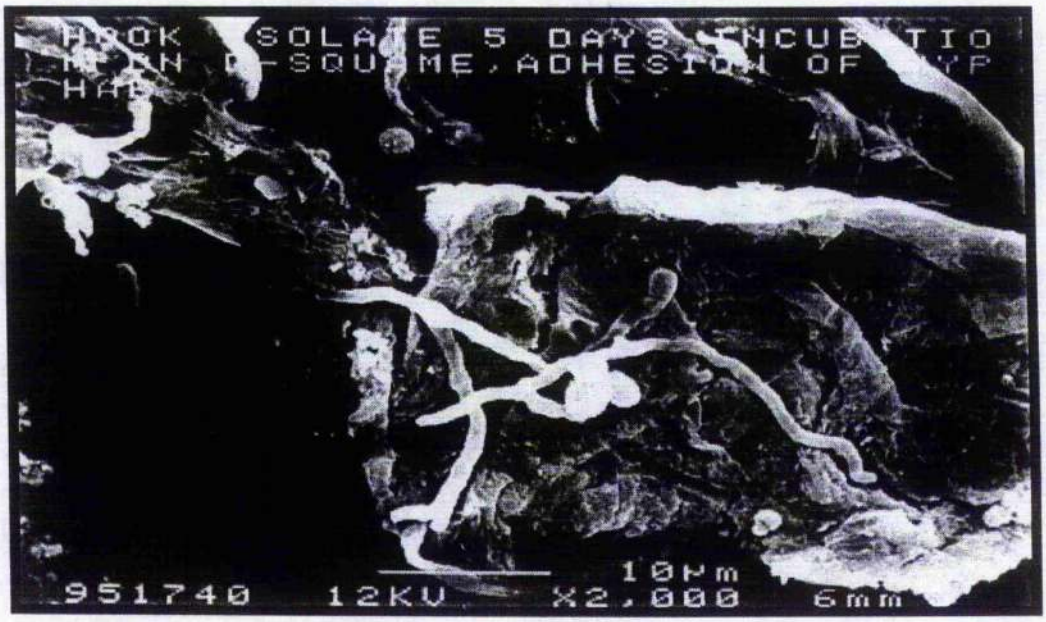

Figure 2.13. Filaments in association with yeast cells. Some filaments are observed to follow the surface topography of the corneocytes, whilst other filaments appear to bury into the sample (X2,000).

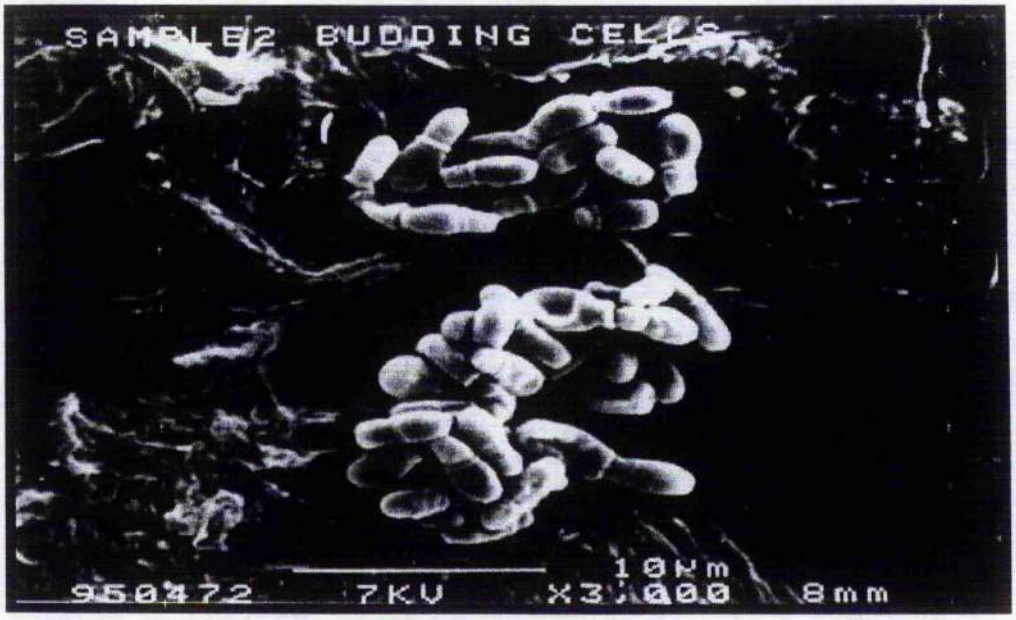

Figure 2.14. Yeast cells observed on nonsupplemented stripped stratum corneum sample. Elongate, oval and budding yeast cells are observed, with no evidence of filament production (X 3,000).

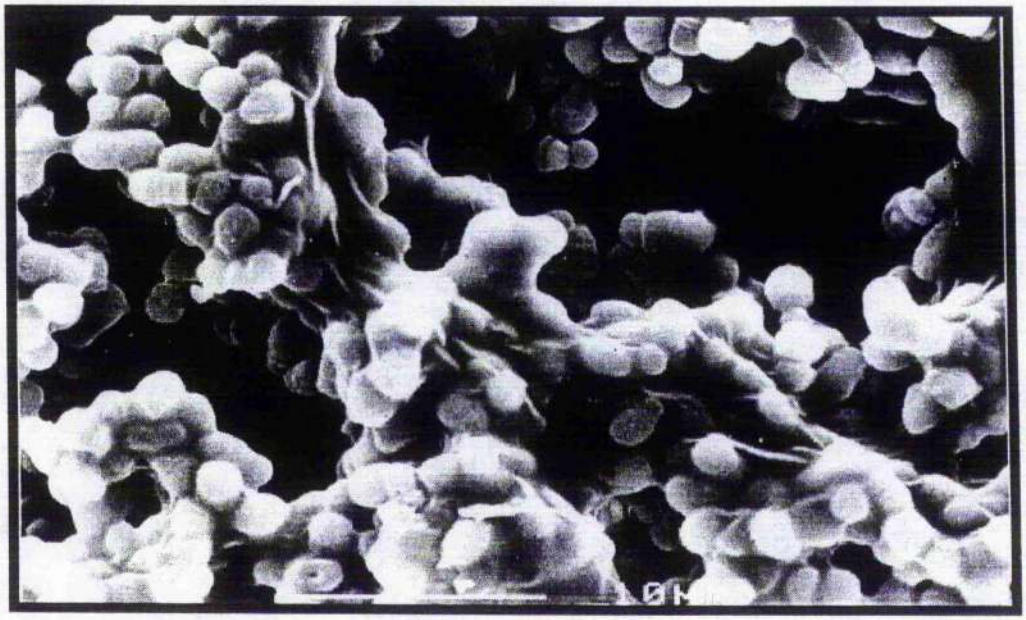

Figure 2.15. *M. furfur* on squalene-supplemented stripped stratum corneum sample. An extracellular coating can be observed to form over clumps of yeast cells (x3,000).

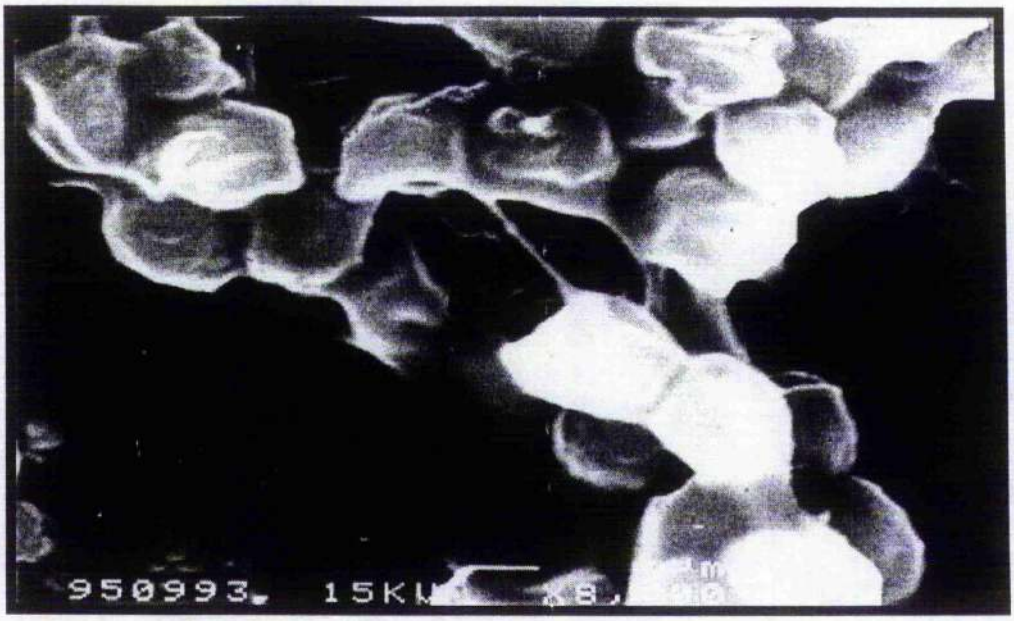

Figure 2.16.. *M. furfur* on cholesterol-supplemented stripped stratum corneum sample. An extracellular coating can be observed to be associated with large clumps of yeast cells (X8,000).

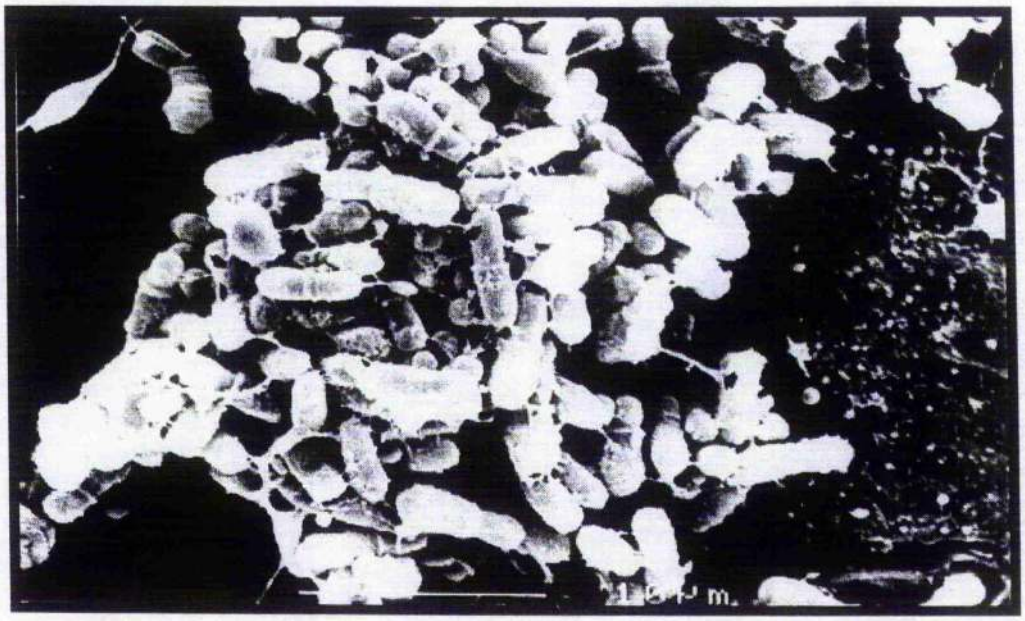

Figure 2.17. Fibrin-like or plasmodesma-like structures associated with the yeast cell surface.These structures could be observed to form attachments from yeast cell to yeast cell as well as to the corneocyte surfaces (x3,500).

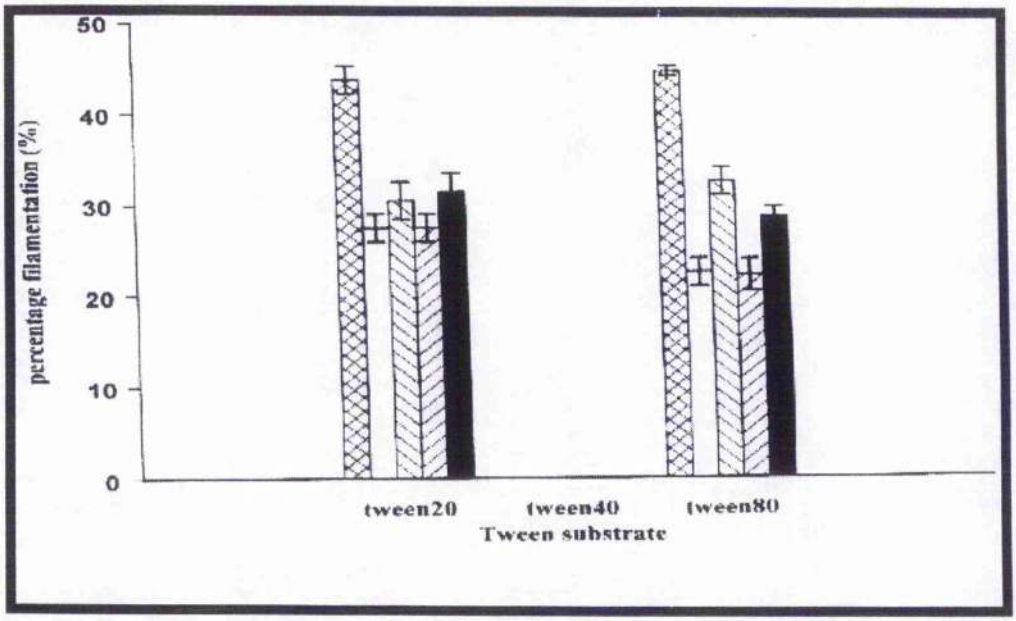

Figure 2.18. Filamentous growth of *Malassezia* isolates in Dorn's medium supplemented with various Tween substrates. ⊠ Hook □GM110 ◪GM340 ◹ L251 ■ D82412

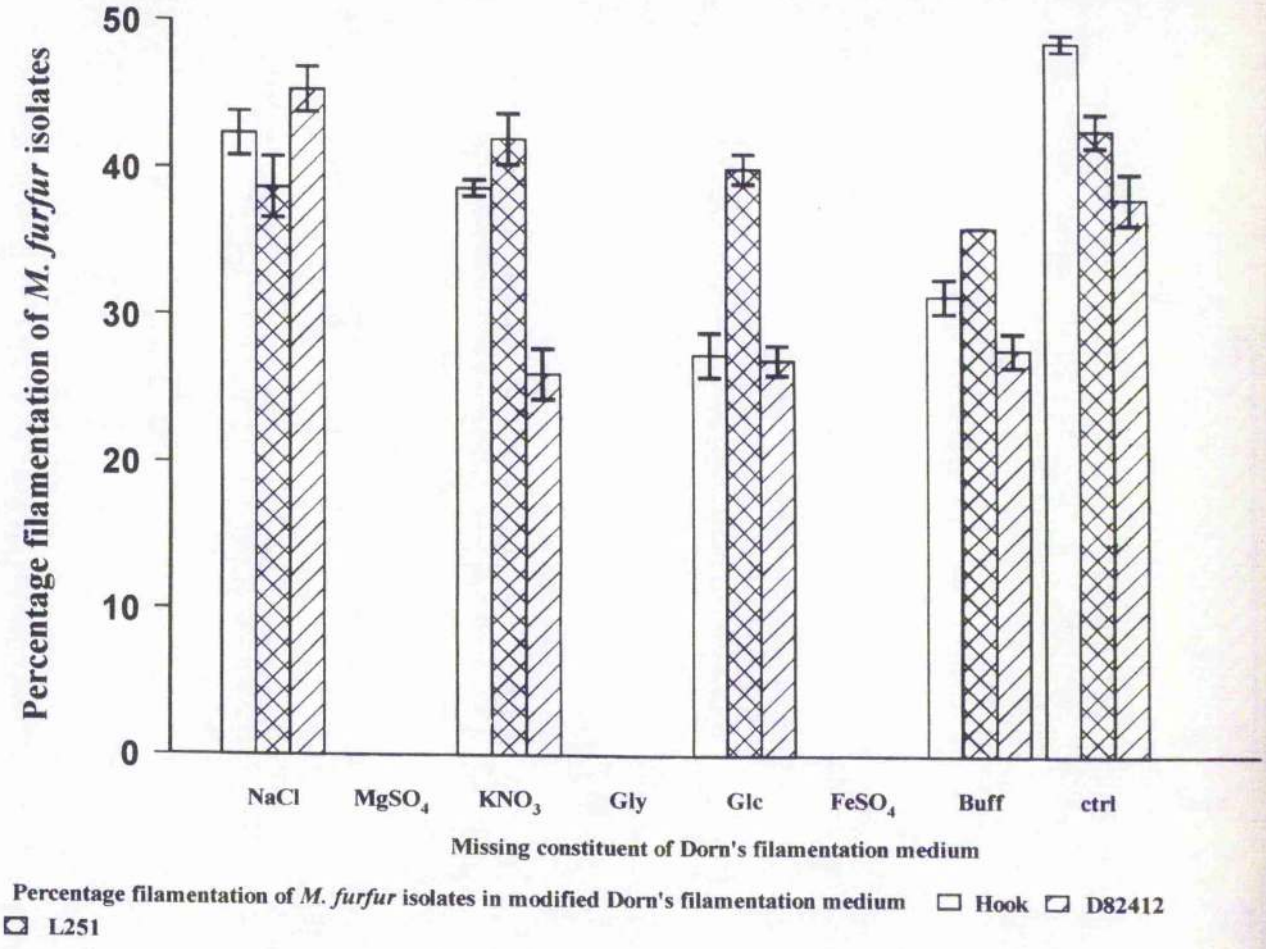

Figure 2.19. This graph represents the effect of omitting each component of Dorn's filamentation medium individually on the effect of filamentous growth of three *M. furfur* isolates. On removal of magnesium sulphate, glycine or iron sulphate the ability to produce filaments was lost for these isolates, which were known to have a propensity to form filaments in complete Dorn's medium. These results are averaged over three separate sets of experiments.

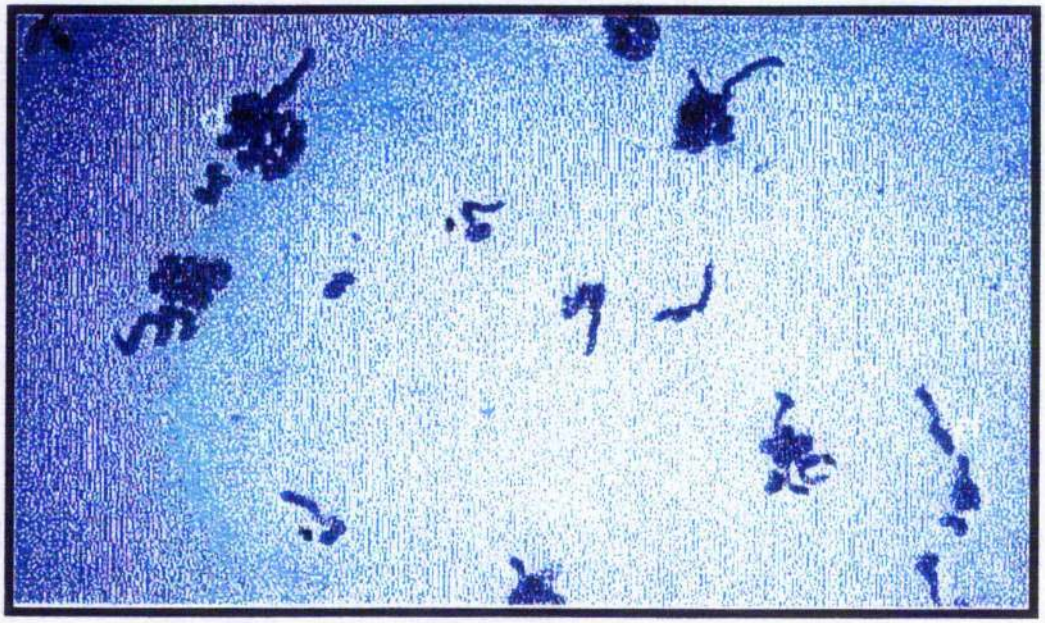

Figure 2.20. Filamentation of *M. furfur* isolate 0333.1 after 5 days incubation at 29°C on YMA supplemented with 8% squalene. Irregular filaments are observed, protruding from various points on the yeast cell surface. Phialospore extrusion is observed. (X400).

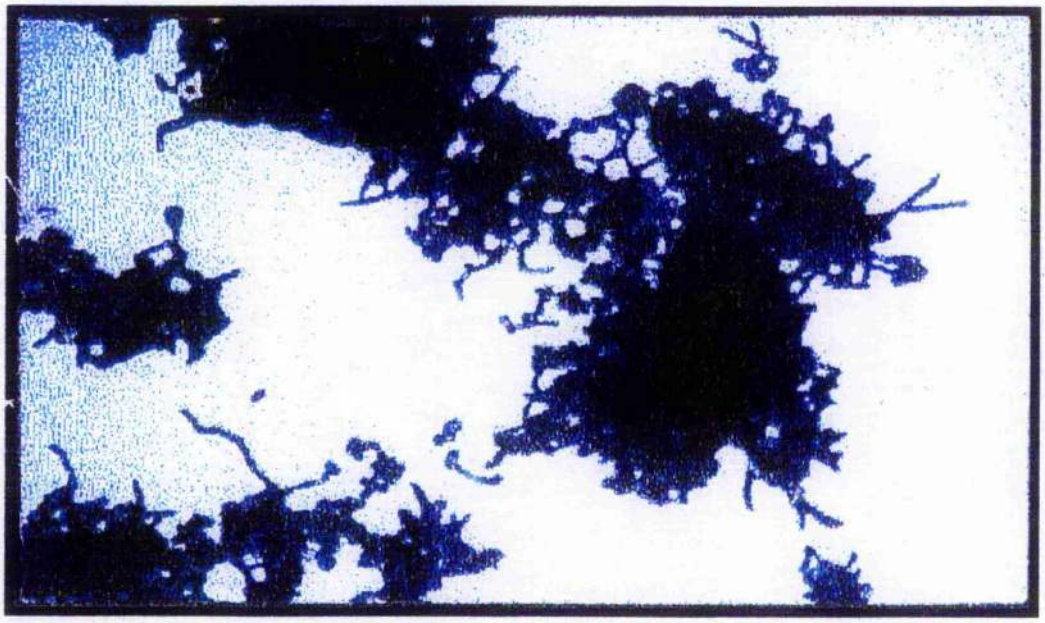

Figure 2.21. Filamentation of *M. furfur* Hook isolate after five days incubation on YMA supplemented with 8% squalene. This isolate showed a higher potential for filament formation in comparison to isolate 0333.1 Branching of irregular filaments and phialospore extrusion is also observed (X400).

supplemented with 8% squalene, *M. furfur* isolates were observed to produce filaments under light and electron microscopy. Filaments under these conditions did not differ from those observed under the other conditions examined (Figure 2.20, Figure 2.21).

2.6 DISCUSSION

In this study, the most promising model of filament production appeared to be lipid- supplemented stripped stratum corneum samples. In such a model, filament production was observed in various *Malassezia* species.

2.6.1 The role of squalene in the yeast-hyphal transition.

Squalene, a sebum lipid, was observed to stimulate filament production in *Malassezia* isolates in the presence of stripped stratum corneum or in solid medium. In broth medium, squalene as a sole lipid supplement was not capable of stimulating growth (not shown). This is probably due to the length of the carbon chain making this hydrocarbon too large to be utilised by *Malassezia* yeast cells for growth under these conditions. Squalene is a key constituent in the ergosterol biosynthetic pathway. It is a carbon 30 isoprene hydrocarbon which can be oxidised by squalene

epoxidase allowing the cyclisation of lanosterol which can then be converted to ergosterol in the endoplasmic reticulum [223]. Ergosterol is the most common fungal membrane sterol and plays a key role in maintaining the integrity of eukaryotic membranes [223]. This quasiplanar molecule stabilises fungal membranes by Van der Waals interactions with the fatty acyl chains of phospholipid molecules. Ergosterol is also known to play a role in membrane-bound enzyme activity and cell wall biosynthesis [223]. In other systems, evidence exists to suggest a role for ergosterol in the yeast-mycelium transformation. Some studies have shown that the ergosterol concentration is greater in the mycelial form of *C. albicans* than in the yeast form [196,223] and ergosterol depletion plus accumulation of 14-methyl sterols blocks hypha formation possibly by steric hindrance exerted by the presence of a methyl group at C-14 which affects membrane fluidity [223]. It is possible that exogenous squalene may be utilised in the formation of ergosterol in preference to the complicated biochemical pathway from acetate to ergosterol. A higher concentration of ergosterol than other membrane sterols has been shown in various fungi to increase membrane fluidity [196,223]. Ergosterol is known to buffer membrane fluidity [222]. There may be a connection between membrane fluidity and filamentation. In *Candida albicans*, growth of germ tubes is known to be aided by a membrane vacuolation process. Gow and Gooday [94] showed that the cytoplasmic membrane of blastoconidia migrates into the germ tube of *C. albicans* and as the germ tube extends, the parent yeast cell is left highly vacuolated with hyphal compartments behind the apical tip becoming vacuolated as cytoplasmic regeneration occurs in the parent cell. This is necessary for the formation of secondary germ tubes and branches. It is possible that exogenous addition of squalene increases ergosterol production and thus membrane fluidity and increased fluidity may aid membrane

vacuolation and hence filament production. It may be important to examine the squalene concentrations in infected areas of patients with pityriasis versicolor to see if there are increased concentrations in these areas compared to noninfected sebaceous sites. This may help to elucidate changes between commensal and pathogenic potential. A study by Chiew and Sullivan [44] showed that exogenous addition of ergosterol prevented germ tube formation in *C. albicans,* although other species such as the plant pathogen *Phymatotrichum omnivorum* can utilise ergosterol in the yeast- hyphal transition [10]. In *Malassezia* species, it may be possible that ergosterol precursors can be utilised in the yeast-hyphal transition. Other precursors such as acetate also require such examination.

2.6.2 The role of cholesterol in the yeast-hyphal transition in *Malassezia* yeasts

A possible role for cholesterol in the yeast-hyphal transition was also suggested. Cholesterol is not a sebum lipid but is found on the skin surface as a product of the decomposition of keratinaceous material. Cholesterol is a secondary fungal membrane sterol and confers membrane rigidity through a process of condensation of the aliphatic polymethylenic chains of phospholipids which leads to a reduction in occupied area of every phospholipid molecule, thus inducing changes in membrane shape and stability [237].

In the stripped stratum corneum model, 1.5% cholesterol (the average skin surface concentration of this sterol) in the presence or absence of oleic acid was capable of inducing the yeast-hyphal transition. Nazzaro-Porro [162] induced

filament production in *P. orbiculare* and *P. ovale*, when a mixture from 0.25 to 2.0% of cholesterol: cholesteryl stearate: glyceryl monostearate (2.0:1.5:2.0) was added to batch yeast morphology agar in the presence of oleic acid [162]. It was suggested that this specific emulsion of cholesterol and cholesterol esters was able to be taken up by the yeast cells stimulating filamentous growth. In this study, cholesterol in the presence of stripped stratum corneum was sufficient to induce filament production. The specific emulsions of Nazarro-Porro [162] did not stimulate the yeast-hyphal transition or growth. It is possible that the importance of cholesterol in the maintenance of membrane integrity may be species-dependent and may dictate the exogenous uptake of cholesterol. The percentage of filament- producing yeast cells under cholesterol-induced conditions was much lower than in the presence of squalene. This may suggest that some mechanisms involved in filamentation of *Malassezia* yeasts may be dependent upon the sterol composition of the membranes and the factors which influence it, similar to the intrinsic relationship between sterol composition and morphogenesis in *C. albicans* proposed by Shimokawa *et al* [206]. Since a role for membrane sterols in the yeast-hyphal transition of *Malassezia* yeasts has been outlined, an examination of the role of the cell membrane in *Malassezia* dimorphic transitions should be examined to see if it is similar to the *C. albicans* model.

2.6.3 Induction of the yeast-hyphal transition in *Malassezia* species in broth culture.

Dorn [63] successfully induced dimorphism in *P. orbiculare* in a culture medium consisting of 0.05M glycine in 0.03-0.06M ammonium phosphate buffer (pH 5.6), salts, glucose and Tween 80. Of the strains tested, 78-85% produced hyphae from up to 8.5% of yeast cells at 29°C. This transition was not observed in any *P. ovale* strains tested. The yeast-hyphal transition was then cited as a possible method to differentiate between the two species [63].

In this study, various species have been observed to form filaments. Although this does not point to inherent problems in Dorn's method, it does suggest that the new classification of *Malassezia* yeasts [97] will highlight filament-producing strains for both species (*P.ovale* and *P.orbiculare*) examined in Dorn's original work. Another difference between this study and the original authors' work was the media component thought to stimulate filamentation. Experiments involving removal of individual media components, showed that filamentation did not occur on the removal of glycine, magnesium sulphate or ferrous sulphate. However when each of these components was individually added to the remainder of the non-stimulatory media components, magnesium sulphate was the only one observed to stimulate filamentation in *M. furfur* isolates. The original authors however, proposed the role of glycine in the yeast- hyphal transition. At concentrations of < 0.05M glycine, filamentation was not observed whereas at concentrations greater than 0.05M, filament production was observed in 0.25-2.9% of yeast cells [63].

Magnesium has been proposed to play a central role in *Candida albicans* morphogenesis. Hyphal growth of *C. albicans* was found to be inhibited in Mg-deficient media and in the presence of metal ion chelators. Walker *et al* also showed

that a sharp peak in magnesium concentration coincided with the onset of germtube formation [226]. This was compared to germ tube-deficient strains which had a lower Mg content and which were unable to accumulate Mg when incubated under germ tube- inducing conditions. The role of magnesium in *C. albicans* germ tube production proposed by Walker *et al* could be linked to the earlier work of Widra *et al* (1964) [233] where germ tube production in *C. albicans* isolates in high phosphate conditions was thought to be due to excess polyphosphates binding high levels of Mg^{2+} causing filamentation . It is possible that a similar system exists in some *Malassezia* species . Similar measurements of intercellular Mg concentration in filamenting and non- filamenting *Malassezia* isolates need to be carried out as well as assessing the difference in number of filament producing cells of *Malassezia* isolates in variable concentrations of Mg and under high phosphate conditions.

Under all conditions examined, the filaments produced from each isolate were long and irregular, very different from those observed in clinical situations and compared to the observations of Nazarro-Porro *et al* [159]. This may be due to a comparatively more nutritious environment compared to *in vivo*. An interesting phenomenon, observed throughout this study was the formation of phialospores, which are ovoid or bottleshaped cells forming single buds, developed from hyphal filaments. In each case, a clearly visible collarette was formed, signifying the parent cell. Tosti *et.al* [218] speculated that this phenomenon only occurred in the deeper layers of the stratum corneum. Filamentous growth was thought to aid penetration whilst phialospore extrusion lead to spores being brought back to the skin surface by epidermal turnover reinitiating the infectivity process [218]. The extracellular coating and plasmodesma formations were also noted by Tosti in biopsy specimens from

lesions of pityriasis versicolor patients. Tosti *et.al* [218] speculated that these structures indicated that the yeast-hyphal transition was present. Civila *et al* [45] also observed phialospore extrusion, but to a greater frequency and did not observe this phenomenon to be confined to particular regions of the stratum corneum . Nazarro-Porro *et al* [159] also noted simultaneous bud extrusion on one side of a filament with filament production at the other end of the same hypha. In the stripped stratum corneum model, this finding was observed on superficial stratum corneum without evidence of penetration which suggests that all the nutrients and signals required to trigger this mode of growth are available at the stratum corneum surface. Evidence in the existing literature suggests that these factors are also available throughout the stratum corneum, aiding penetration [218].

A low potentiality of *Malassezia* yeasts to undergo the yeast-hyphal transition *in vitro* has been observed in this study. From 1886, workers such as Hansen have stated that this potentiality is highly variable among yeasts and in general the yeast -hyphal transition is never complete as yeast forms will invariably be found in filamentous cultures [103]. Hansen's work in this field was pioneering and his observations on the yeast hyphal transition in *Saccharomyces* species have become an integral part of the modern literature.

Another variation on Dorn's work was examined [63]. By varying the Tween supplement whilst all other conditions were kept constant, filamentation occurred in the presence of Tween 20 and Tween 80, but not in the presence of Tween 40. Tweens are derivatives of various fatty acids with questionable purity. On average Tween 80 consists of approximately 75% oleic acid, balanced with linoleic- palmitic-

and stearic- acid. Tween 40 is of approximately 90% purity, balanced with stearic acid, and Tween 20 is approximately 55% lauric acid, being balanced with myristic, palmitic and stearic acid. It is possible that the impurities in Tween 20 and Tween 80 may be the cause of the yeast-hyphal transition. Notably, Tween 40, which did not stimulate filamentation, has the highest percentage purity. Possibly the transition we have observed is due to the contaminating lipids present in these preparations. Similar observations were made by Shifrine and Marr [205]. when they discovered that *P. ovale* would not grow in the medium supplemented with pure oleic acid on its own as a lipid source, but responded to the saturated fatty acids myristic and palmitic acid which were found in crude oleic or catalytically hydrogenated crude oleic acid when these oleate sources were used as the sole lipid supplements in their study .

2.7 CONCLUSIONS

This study has shown that addition of membrane sterols and squalene, the precursor of ergosterol to stripped stratum corneum biopsies will stimulate the yeast-hyphal transition of some *Malassezia* isolates.. The link between magnesium sulphate and filamentation in *Malassezia* yeasts may indicate that second messenger systems may also play a role in filament production. The production of filaments may not be used as a parameter for distinguishing between species although they provide a useful indicator of pityriasis versicolor, when isolated from lesional sites. Species dependent and environmentally dependent parameters are involved in dimorphic regulations and this transition is controlled by more than one independent parameter alone .

CHAPTER3: COLONISATION OF LIVING SKIN EQUIVALENTS BY *MALASSEZIA* YEASTS.

3.1 SUMMARY

Initial colonisation events and yeast-hyphal transformation by *Malassezia furfur* (*M. furfur*) were observed using a living skin equivalent (LSE) model for growth. Yeast cells were inoculated onto the LSEs which were incubated in CO_2-independent media at 37°C for variable lengths of time. Assessment of fungal growth and invasion was by light- and scanning electron-microscopy (SEM). Viability counts of *M. furfur* were determined by a method of washing and serial dilution. Yeast cells had retained their viability and increased in number approximately two-fold over a four day period of incubation. Yeast-to-hyphal transition was not achieved in this model. Random destruction of the uppermost layers of the stratum corneum through loss of cohesion was observed in the presence of *M. furfur*. Living skin equivalents therefore appear to be a promising model for mechanisms of growth of cutaneous organisms.

3.2 INTRODUCTION

Living skin equivalents (LSE) can be reconstructed *in vitro* and provide useful models for assessing *in vitro* phenomenon observed *in vivo*.

The idea of composing a collagen lattice dermal equivalent was developed from the work of Bell *et al* [15]. Bell developed a skin equivalent model [15,16]

which consisted of a dermal component made up of fibroblasts in a collagen matrix that is contracted and modified by the resident cells, and an epidermis that consisted of keratinocytes obtained from suction blisters or skin biopsies, which were seeded onto the collagen lattice, where they stratified and differentiated to form a multilayered, keratinising epidermis containing resident skin cells such as desmosomes, tonofilaments and hemidesmosomes [16]. Common to other methods of skin equivalent construction is the culture of keratinocytes at an air-liquid interface. Boyce and Hansborough [30]developed a method for constructing skin equivalents for use in full thickness skin grafting which consisted of plating mitotic keratinocytes onto an acellular sheet of collagen and chondroitin-6-sulphate dermal skin replacement [30]. The mitotic keratinocytes, derived from tissue culture were observed to attach rapidly to the dermal membranes and become confluent to form a continuous sheet of epithelium, which could then be promoted to stratify and differentiate to a moderate degree.

Bell's skin equivalent model [16] has been exploited by workers such as Coulomb *et al* in the investigation of the activity of pharmacological agents [48]. In comparison to the previous monolayer models used in the study of pharmacological agents, these skin equivalents contain an epidermis consisting of a stratum corneum and stratum granulosum, which include keratohyalin and membrane-coating granules, providing a more realistic model for assessing pharmacological activity. The models can also be used to study cell responses at the tissue and organ levels *in vitro* [15,16].

The close resemblance to the properties of skin *in vivo* possessed by these models make them ideal models for assessing such factors as percutaneous absorption,

the effect of UV radiation, and toxicity testing, as well as basic research, and also have other applications such as in epidermal grafting for extensive burn wounds and chronic ulcers [168].

In each of the skin equivalent methodologies, keratinocytes were grown in tissue culture and were obtained from suction blisters and skin biopsies [168,142]. Due to the culture of keratinocytes being a time-consuming process with a low multiplication efficacy [186], a method was developed in which keratinocytes could be obtained from neonatal foreskin and be seeded directly onto the collagen lattice dermal equivalent [183]. This has been shown to be a less time consuming process, where an epidermis forms with the same properties as *in vitro* epidermis formed from tissue cultured keratinocytes. This method was exploited by Rashid *et al* in an investigation of the activity of the antimycotic agent terbinafine against the dermatophyte *Trichophyton mentagrophytes* [183]. The work of Rashid *et al* provided pioneering experimental work into aspects of fungal invasion of skin using a novel model. Previous to this, studies of dermatophyte infection of *in vitro* skin models were limited. Blank *et. al* [26] studied the growth of dermatophytes on pieces of prepuce obtained from neonatal circumcision, on sera-containing agar medium. As batches of LSEs can be grown *in vitro* in a uniform manner, they provide a superior method of examining stratum corneum invasion as external variation is limited in comparison to methods using dissected, intact viable tissue. To date, no other published work has assessed fungal invasion into stratum corneum tissue using a living skin equivalent.

In this study, LSEs were used to assess colonisation and invasion of stratum corneum by *M. furfur*. Most other studies of skin pathogens have been limited. This is

due to the lack of satisfactory models to study early skin colonisation events. The work of Faergemann provided an excellent opportunity to study the effect of various factors on the dimorphic transition and the behaviour of the organism in its natural habitat [76]. Other methods of studying cutaneous pathogens include the use of steridrape stripping for isolating stratum corneum cells which in turn can be used for germination assays of dermatophyte fungi [4] as well as the D-squame biopsy method used in section 2.4.3. The lack of sophisticated models employed for *in vitro* assessment of *M. furfur* pathogenicity prompted the need for study of *Malassezia* on a living skin equivalent. Due to the absence of human serum, this model provided an ideal system in which to study the initial events in skin colonisation by *Malassezia*.

3.3 AIM OF STUDY

The aim of the study was to examine growth and colonisation of LSEs by *M. furfur in vitro*, in order to elucidate pathogenic mechanisms involved in dandruff and seborrhoeic dermatitis, and to observe whether colonisation of skin equivalents alone was enough to induce the yeast-hyphal transition in isolates known to have a filamentous capacity.

3.4 METHODS

3.4.1 Organisms and stock cultures

An *M. furfur* isolate (Hook) was selected for this study, due to its propensity to form hyphae. This isolate was obtained from a scalp psoriasis study patient at the Western Infirmary, Glasgow. Stock cultures were maintained in cryobanks (Mast Diagnostics, Merseyside, UK), and grown on modified Dixon's agar at 37°C.

3.4.2 Preparation of inoculum

Pure suspensions of *M. furfur*, prepared from four day old cultures harvested from modified Dixon's agar, were made up in Dulbecco's phosphate buffered saline (PBS). Yeast cells were counted in a haemocytometer and a 20µl inoculum of a $3x10^6$ yeast cells ml^{-1} suspension was used for each LSE infected.

3.4.3 Preparation of dermal equivalent.

Type 1 collagen was extracted from rats tail tendons in 0.5M acetic acid and precipitated in an equal volume of 10% w/v NaCl, redissolved in 0.25M acetic acid, with the final solution being adjusted to 3mg collagen ml^{-1}. Collagen gels were prepared by mixing 7 volumes of collagen solution with two volumes of a mixture of

10x Eagles's minimal essential medium (MEM):0.34M NaCl (2:1 v/v), and the pH finely adjusted to 7.2 with NaOH. One volume of foetal calf serum (FCS) containing 7.5 x 10^5 human forearm fibroblasts/10ml of gel mixture was added, mixed thoroughly and 4ml aliquots pipetted into 35mm petri dishes. The gels were allowed to set at 37°C for 15 min, after which 2ml of MEM supplemented with 10% FCS was added, the gels detached from the dishes, and incubated for nine days at 37°C in a humidified atmosphere of 5% CO_2 in air, with a medium change every three days. The gels at this stage were highly contracted, with the fibroblasts adopting a spindle shaped morphology [183].

3.4.4 Keratinocyte culture

Neonatal foreskins were cut into 3mm strips after removal of subcutaneous fat, floated on to 0.5% w/v dispase in PBS, and the tissue incubated overnight at 4°C. The epidermis was peeled off with forceps, finely chopped with a scalpel into fine pieces and the keratinocytes were dissociated by treatment with 0.05% (w/v) trypsin, 0.02% (w/v) EDTA in Ca^{2+}- and Mg^{2+}-free PBS for three minutes with pipetting to obtain a predominantly single cell suspension. The cell suspension was passed through sterile gauze, centrifuged (4min, 400g), resuspended in Dulbecco's modified MEM supplemented with 10% FCS to inactivate the trypsin, and added to contracted collagen lattices in a 24 well multidish. Following incubation at 37°C for five days as submerged cultures, the gels were then raised to the air-liquid interface by placing

them on coarse sintered glass disks, and incubated for a further seven days at 37°C, at which time a highly stratified epidermis was formed.

3.4.5 Assessment of LSE colonisation by *M. furfur*

Gross examination was carried out to assess fungal growth. For light microscopy, the LSE was fixed in 4% (v/v) formaldehyde and dehydrated and embedded in paraffin wax for haemotoxylin and eosin (H and E) and Periodic Acid Schiff (PAS) staining after incubation for the appropriate time intervals.

For scanning electron microscopy, skin equivalents were fixed in 2.5% gluteraldehyde and dehydrated in graded ethanol solutions, substituted with liquid CO_2, dried in a critical point dryer, mounted on 10mm aluminium stubs (Jeol), sputter coated with gold, and examined in a JSM 6400 scanning electron microscope.

3.4.6 Viability counts of *M. furfur* on living skin equivalents.

Every 24h, duplicate LSEs were removed from incubation. These LSEs were placed in separate sterile containers containing 5ml PBS and shaken at 150 rev/min for 30 min to remove any nonadherent cells. The resulting suspensions of non-adherent cells were then serially diluted from 10^{-1} to 10^{-3}. Previous experiments indicated that

greater dilution did not give representative numbers of colonies on viable colony count assessments *i.e.* there were not enough colonies on each plate to give an accurate indication of the number of viable cells. Aliquots of 100µl of each dilution were plated out on Dixon's agar. Agar plates were then incubated at 37°C for three days. Colonies were counted and hence the number of non-adherent viable yeast cells per ml could be determined.

3.5 RESULTS

3.5.1 Histological examination of living skin equivalents

Vertical histological sections of an uninfected LSE revealed a multilayered epidermis consisting of stratified squamous epithelium with the upper layers showing stratification whilst the basal and middle epidermal layers contained polygonal or pyramidal (unflattened) cells with central nuclei. Mitotic activity, keratohyalin granules, horny layer formation were also observed, giving an indication of the comparability of these models to human skin tissue, and supporting their use in this study (Figure 3.1).

3.5.2 Assessment of LSE colonisation by *M. furfur*

Yeast cells colonised the upper layers of the stratum corneum of the LSE following three days incubation at 37°C (Figure 3.2). Filamentation was not observed, but loss of stratum corneum cohesion was noted in the presence of *M. furfur*, which appeared to become progressively more apparent compared to an uninoculated control (Figure 3.3). Other nucleated cells appeared in these upper layers of the stratum corneum (which were also observed in control models), which may indicate a parakeratotic nature to these models, but are more likely to remain in the upper keratin layer of the stratum corneum as a consequence of the keratinocyte seeding process. A control model incubated for the same length of time showed no colonising yeast cells or random tissue destruction. In contrast, a uniform detachment of the uppermost horny layers was observed, suggesting that colonisation by the yeast cells was affecting the structure and cohesion of the stratum corneum (Figure 3.3).

3.5.3 Viability counts

M. furfur was capable of viable growth on these models (Figure 3.4). Increasing numbers of colonies on the viability plates showed proliferation of *M. furfur* over a 4d period. The yeast cells were easily removed by gentle agitation in PBS suggesting that adherence mechanisms are poor but this proved convenient, as it provided a very accurate method of assessing growth and viability in this model. From the viability

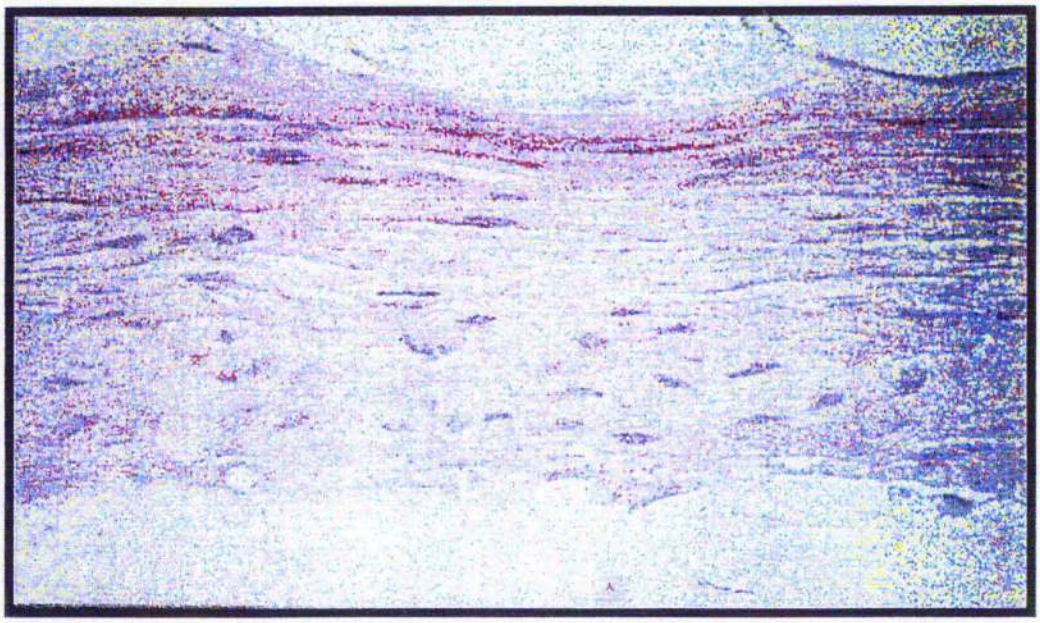

Figure 3.1. Stratification of living skin equivalent after 8 days incubation at 37°C at an air-liquid interface. Note the stratification of the skin equivalent, revealing many simliaritieis to human skin tissue. (H&E, X400).

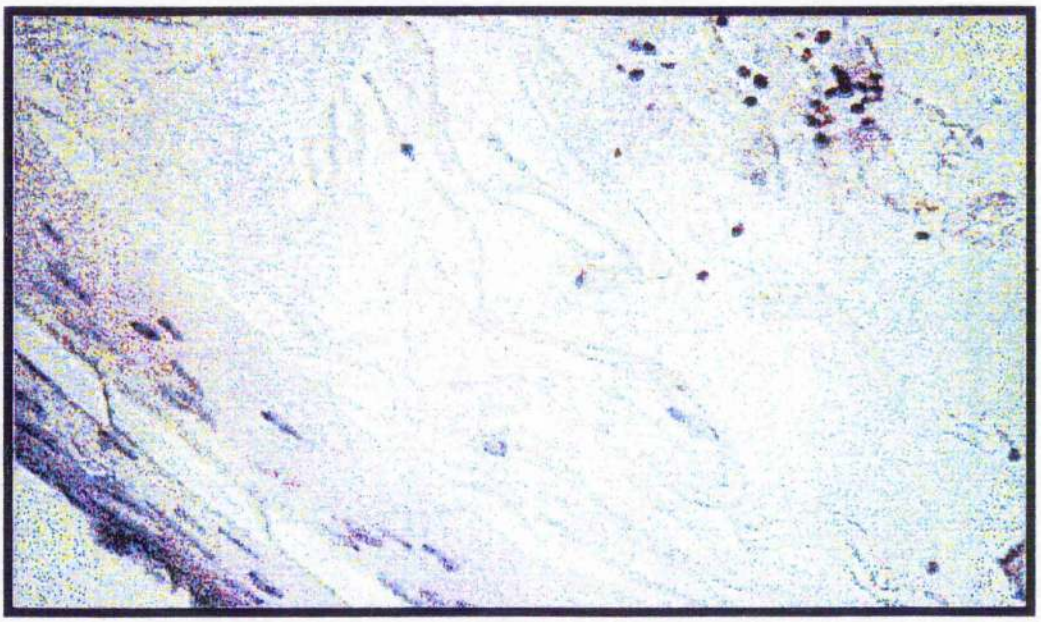

Figure 3.2. Colonisation of the upper layers of stratum corneum tissue on a living skin equivalent after three days incubation at 37°C (PAS, X400).

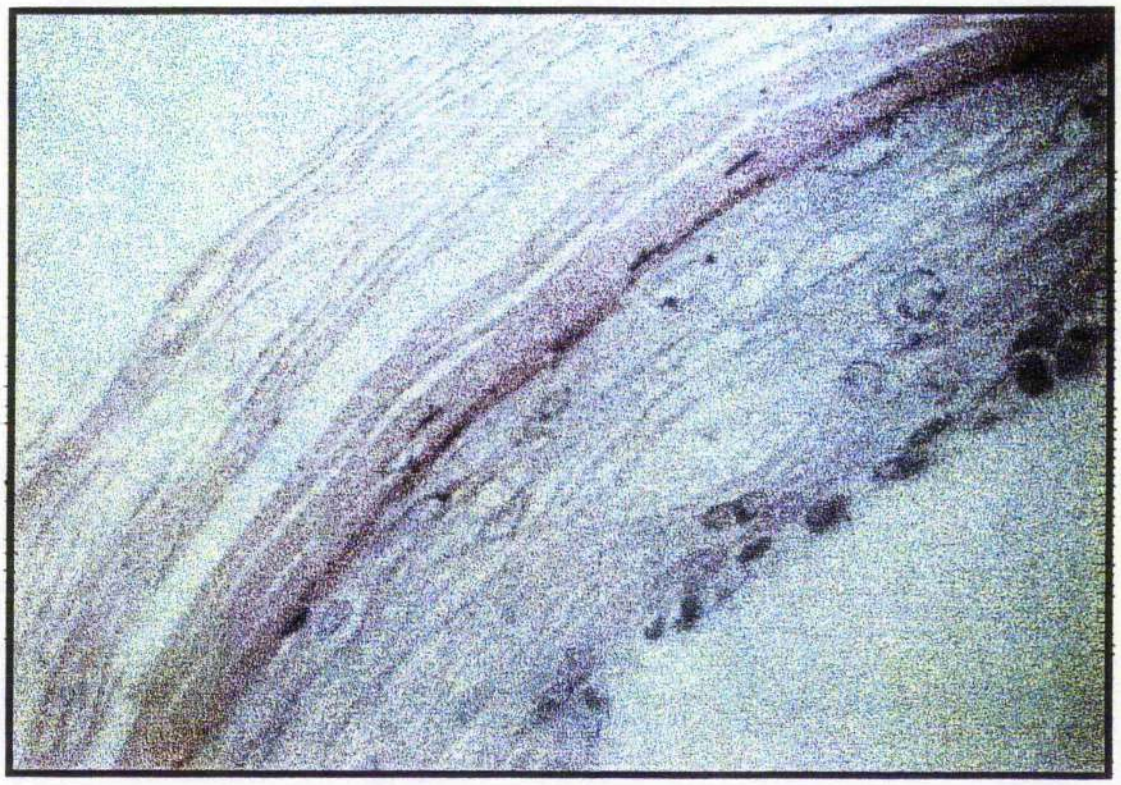

Figure 3.3. After 3 days incubation at 37°C, an uninoculated control model showed uniform detachment of the surface layers of the stratum corneum, in comparison to the random destruction observed in Figure 3.2.

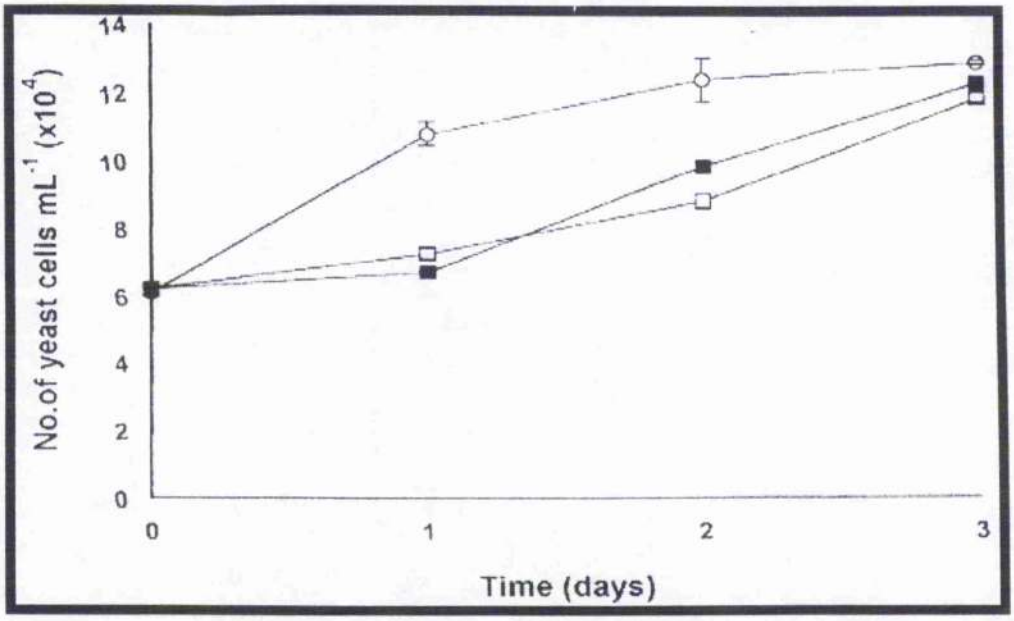

Figure 3.4. Growth of *M. furfur* on a living skin equivalent. In three separate experiments with duplicate readings, an increase in *M. furfur* growth was observed. For each experiment, using Student's T -test on lognormalised data, the increase in growth was observed to be highly significant ($P<0.001$). Each dataset line on the graph represents repeated experiments.

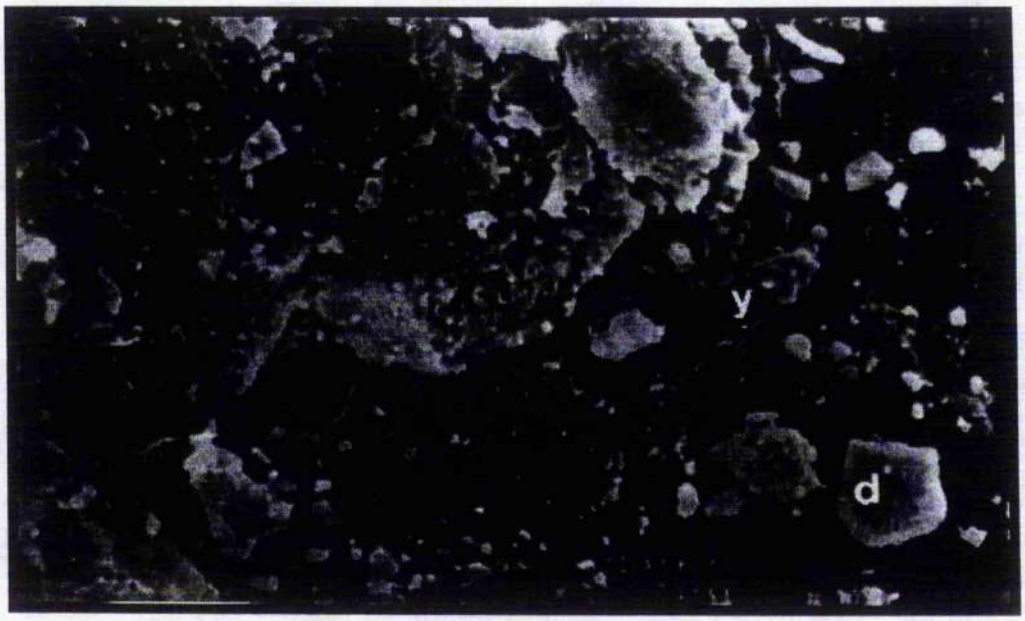

Figure 3.5. Scanning electron micrograph of yeast cells (y) and stratum corneum debris (d) on the surface of the skin model (X1,300).

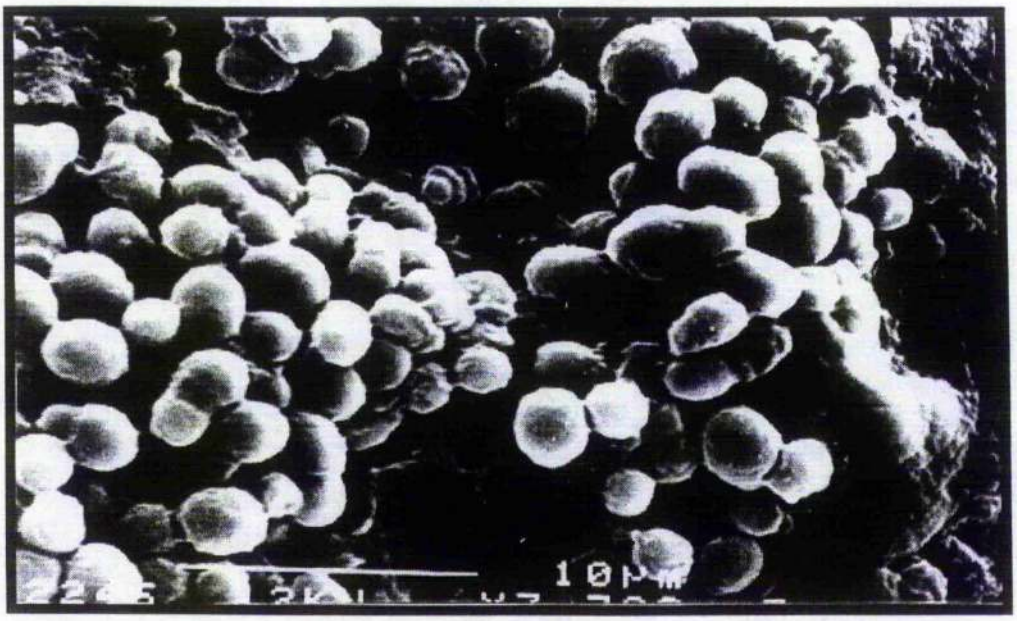

Figure 3.6. Scanning electron micrograph of living skin equivalent colonisation by *M. furfur*. Yeast cells apparent on the upper surface of the stratum corneum. Tissue destruction is also apparent (X3,300).

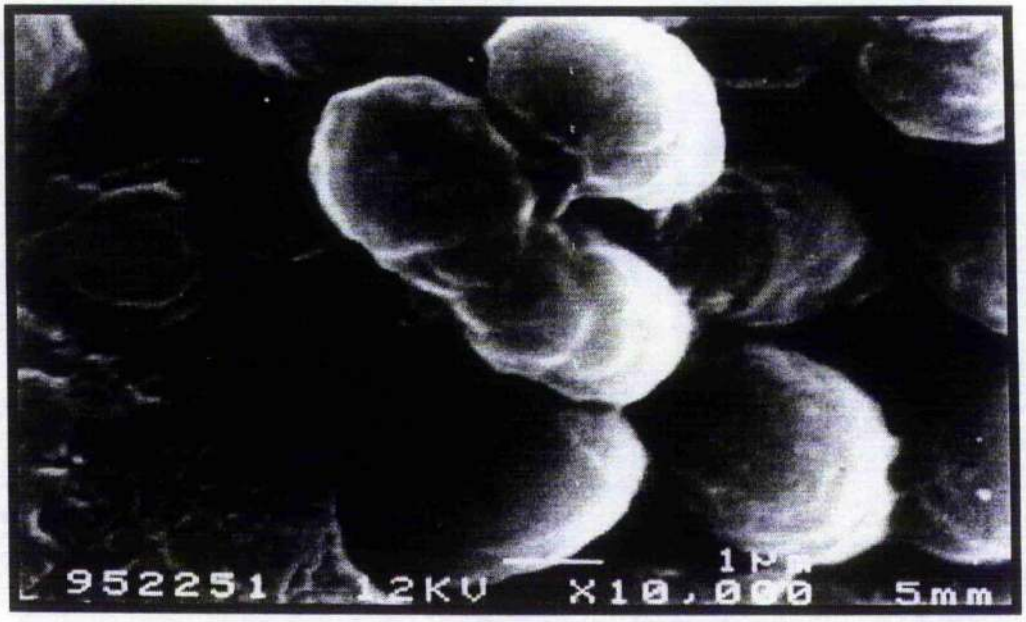

Figure 3.7. Scanning electron micrograph of *M. furfur* yeast cells with a lipidic-like cell surface coating. Yeast cells are observed burying into corneocyte surfaces (X 10,000).

counts, the number of viable yeast cells ml^{-1} could be determined, with almost an 100% increase in growth observed.

3.5.4 Scanning Electron Microscopy

On SEM microscopy, yeast cells were again observed to be colonising the uppermost layers of the skin model. Destruction of stratum corneum tissue observed under light microscopy was again noticeable under SEM examination (Figure 3.5), yeast cells appearing to be confined to the surface although penetration to the deeper layers of the stratum corneum was not obvious (Figure 3.6). As observed in the stripped stratum corneum model (section 2.5.1), yeast cells were observed to have a lipidic-like coating over the cell surface as yeast cells embedded into the surfaces of corneocytes. However, the extracellular coating was not as extensive as that observed in the stripped stratum corneum model (Figure 3.7.).

3.6 DISCUSSION

In this study, previous methods of LSE construction [16,48] were adapted by a direct seeding of neonatal keratinocytes onto the surfaces of contracted collagen lattices, to provide a novel method for investigating stratum corneum colonisation by *M. furfur*. Because of the absence of human serum in these models, the LSEs were thought to be

an excellent method to study colonisation and growth of *M. furfur*. Colonisation, and morphology were examined using light and electron microscopy, and could be compared to the observations made in the stripped stratum corneum model.

3.6.1 Viable growth of *M. furfur* on LSE models

This is the first study using the LSE to study *in vitro* colonisation of stratum corneum by *Malassezia* yeasts and was undertaken to examine the establishment of growth of *M. furfur* in a skin surface environment.

Viability counts consistently revealed that viable growth of *M. furfur* on LSE epidermal equivalent occurred. These indicated that the LSE model contained enough nutrients and signals required for growth of *M. furfur*. Usually *in vitro*, a lipid supplement must be added to the culture for growth of *M. furfur*. In this model, no lipid supplement was added to the LSE surface. LSEs also lack the presence of sebaceous glands which are epidermal appendages that lie in the dermis, yet viable *M. furfur* growth is observed. The sebaceous gland produces the majority of lipids and fatty acids, which are found on the skin surface, by secretion of sebum [67]. The rest of the skin surface lipid mixture is mainly composed of intracellular lipids such as cholesterol,and free fatty acids In the LSE, lipids are present from the FCS used in the preparation of the LSEs, and from the keratinisation process as the epidermal equivalent differentiates. The level of lipid contribution from these sources has not

been measured in the LSE, however it appears that through the stratification process and the addition of FCS, the LSE has a large enough lipid spectrum to sustain the growth of *M. furfur*.

The transition from yeast-phase growth to hyphal-phase growth was not observed in this experimental model suggesting that other factors in addition to initial colonisation are required to induce this transition. In section 2.5.1, it was indicated that when various sebum components such as squalene and skin surface lipids such as cholesterol were exogenously supplied to *M. furfur* growing on samples of stripped stratum corneum at 29°C in a humidified environment for 5d, a change from yeast to hyphal growth was observed in 15-75% of the yeast cells observed. A squalene supplement had a higher propensity to induce the yeast -hyphal transformation in comparison to cholesterol in the stripped stratum corneum model. Squalene is a sebum lipid whereas cholesterol is a skin surface lipid formed from keratinocyte decomposition [67]. It is most likely that cholesterol rather than squalene may be present on the LSE surface, but not at a high enough concentration to stimulate the yeast-hyphal transition. An assessment of the actual lipid composition in the LSE has yet to be examined. It is possible that the various skin surface lipids are not present in these skin models at levels similar to those *in vivo* at typically infected sites of the body. It is also possible that by altering environmental factors such as humidity, the LSE lipid spectrum can be manipulated .It is important to look at the addition of squalene and other sebaceous lipids to these models to observe whether growth is enhanced and the yeast-hyphal transition is induced. Some investigators believe that the yeast -hyphal transition occurs due to factors that are host-related more so than organism-related. For example, McGinley *et.al* [150]showed that immunosuppression

from hypercorticosteroid therapy may predispose to tinea versicolor [150] and it is accepted that genetic and constitutional susceptibility play a role. There is also evidence of other underlying disorders which may encourage the pathogenic propensities of the organism [121].

3.6.2 Histological observations

Loss of cohesion and a slight swelling of the stratum corneum was observed. These observations were confined to the uppermost surface layers of the stratum corneum of the LSEs, in the presence of *M. furfur*. There was no evidence of any yeast cell or hyphal penetration into deeper layers of the stratum corneum as observed in pityriasis versicolor lesions . Tosti *et al* [218]noted that in diseases such as pityriasis versicolor, a high concentration of *Malassezia* yeast cells and filaments were found within the upper two thirds of the stratum corneum, destroying the upper part of the layer with swelling, loss of cohesion and cracking. In this study, similar deterioration was observed although the penetration of the horny layer was observed only in the superficial layers, not as deep as observed by Tosti *et.al* [218], which may indicate that other host factors are involved in further penetration and *Malassezia* pathogenicity. In other studies of seborrhoeic dermatitis, yeast cells were often observed clustered in layers parallel to the skin surface with underlying clumps of parakeratotic cells and spotty accumulation of inflammatory cells. In the LSE model there was no evidence of parakeratosis. It was also observed that the pattern of distribution of the yeast cells was different from the patterns observed in normal skin.

The location of *M. ovalis* was almost exclusively limited to the follicular ostia in normal skin, but was more widespread in the intradnexal epidermis in seborrhoeic dermatitis [172]. Dotz *et al*, [64]examining the biopsy specimens from lesions of hyperpigmented tinea versicolor noted that the fungal load was confined to the upper regions of the keratinised material with little evidence of invasion down to the basal layer of the stratum corneum, which is reflected in this LSE study.

In this study, an extracellular coating over clumps of yeast cells was observed under SEM. Although poor adherence mechanisms were noted in the LSE model, such coatings are most likely involved in adherence mechanisms. Studies carried out by Faergemann *et al* [73] and Schectman [200] could not establish a link between pathogenicity and adherence to keratinocytes, as no differences in adhesion were noted between strains isolated from HIV-positive patients and non-HIV-positive patients with *Malassezia* infections. Schectman assessed adherence of *Malassezia* yeast cells to human keratinocytes using a double sided Sellotape method. This was seen as a model for *in vivo* adherence, approximating the conditions found on the skin surface [200]. *Malassezia* strains isolated from HIV-positive patients with seborrhoeic dermatitis did not differ in adherence properties in comparison to strains isolated from non-HIV positive seborrhoeic dermatitis patients. In addition, no relationship between the severity of seborrhoeic dermatitis and *in vitro* adherence to human keratinocytes could be elucidated [200]. This is in comparison to *Candida* species in which there are clear correlations between adherence and virulence [65], and dermatophytes, where adherence, germination of arthroconidia and penetration of the stratum corneum are requirements necessary for invasion of dermatophytes [71].

An earlier study by Faergemann [73] showed that for five strains examined, no differences in adherence to washed epithelial cells was observed between strains but adherence was observed to increase with time and inoculum size. This may suggest that if the inoculating culture had been increased in fungal load we may have observed more attachment to the epidermal surface of the LSEs. Fungal load has also been assessed in correlation with severity of seborrhoeic dermatitis. Heng *et al* [106]indicated a correlation between the density of *P. ovale* and the clinical severity of seborrhoeic dermatitis both before and after therapy with a precipitated sulphur/salicyclic acid shampoo, which supported the theory that *Pityrosporum* (*Malassezia*) yeasts contribute to the pathogenesis of seborrhoeic dermatitis. The observations in this model do not elucidate all the mechanisms involved in the pathogenicity of seborrhoeic dermatitis and dandruff, although further work using LSEs to examine a variety of isolates and inoculum size may be beneficial in this respect. However, these results, in conjunction with the existing literature suggest that other host- related factors are involved in the pathogenicity of these disease states.

Host immune response is thought to be a key factor in the establishment of *Malassezia*-related infections. Pierard-Franchimont *et. al* [172] in 1995 showed that *Malassezia* yeasts (*M.ovalis*) positively reacted to the antibody to factor XI suggesting that a factor XI-related transglutaminase is present in fungal walls, which could act as an adhesion molecule to biological substrates. Kieffer *et al* [125] also found in patients with seborrhoeic dermatitis, no evidence of immune reactions to *P. ovale.* However, the number of suppresser T-cells were increased, thereby indicating a possible immunologic disorder in this disease. Because of the limited knowledge as to

the exact contributory factors in the pathogenesis of *Malassezia*-related infections, this model allowed us only to assess factors related to the fungus itself.

Apart from this, few other fungal factors have been elucidated in the pathogenicity of *Malassezia* yeast infections, in particular in seborrhoeic dermatitis. Destruction of the stratum corneum in the absence of hyphal filaments may suggest that the destruction, adherence invasion at initial stages may be due to the *M. furfur* releasing some enzyme capable of breaking down the stratum corneum. For example, the dermatophytes liberate enzymes called keratinases which aid breakdown of keratinised material. Such enzymatic factors have not been investigated in *Malassezia* species. However, it is not generally thought that *Malassezia* produce destructive keratinases.

3.6.3 Comparison of LSE model and D-squame stripped stratum corneum model in the investigation of *Malassezia* pathogenicity.

In comparison to the skin biopsy D-squame method, LSEs provide models with uniformly grown epidermis which are non- infected by resident microflora. By using LSE models, factors such as topical treatments of the epidermis surface and washing procedures can be standardised, whereas obtaining stratum corneum samples by D-squame biopsy from individuals relies on the investigator standardising such procedures with other individuals involved in the study, leaving more room for experimental error. However, the D-squames have other advantages over the skin

model. In the first instance, sampling from infected individuals can be carried out as well as samples from normal skin. These disks can be visualised under the light microscope because a full thickness of epidermis is not recovered. Although the D-squame biopsies will not contain sebaceous glands, they will contain sebum which has been released to the skin surface from the sebaceous gland, which may be an advantage. The LSEs would have to be altered by varying external factors such as incubation humidity, to provide a spectrum of lipids similar to those in human skin. As the LSE model does not contain sebaceous glands it is likely that the addition of synthetic sebum to the surface of the LSE would be an easier method of changing the lipid environment. In all, the two models used in conjunction should provide a broader picture than either model used in isolation.

3.7 CONCLUSION

This is the first study in which living skin equivalents have been used as an *in vitro* model to assess the pathogenicity of *M. furfur* yeasts. The confinement of yeast cells to the uppermost layers of the LSE stratum corneum, and the absence of filament induction show close similarities to mild cases of seborrhoeic dermatitis or dandruff. Loss of cohesion in the uppermost stratum corneum layers were observed although parakeratosis did not appear to occur as in severe seborrhoeic dermatitis cases. However, the lack of deeper penetration into the stratum corneum of the LSEs is different from observations made in severe cases of dandruff. Lack of deeper invasion in the LSE may be due to a faster cell turnover in the LSE than *in vivo* or the lack of sebaceous gland lipids and therefore a different lipid distribution *in vivo* than *in vitro*

of lipids beneath the skin surface. Because there was no evidence of the transition from yeast-phase growth to filamentous growth in the LSE, we were unable to observe whether deeper penetration of the LSE stratum corneum was possible in a manner similar to that observed in pityriasis versicolor. However, as these models provide a growth substrate for *Malassezia* yeasts, it is possible that LSEs may be used to examine the manipulation of *M. furfur* growth and viability by the addition of exogenous materials. The LSE also provides a model that is an alternative to an animal model for the investigations of cutaneous infections although its main use will most likely be found in the screening of antifungal drugs in a predictive manner, under carefully monitored conditions.

Chapter4: Growth of *Malassezia* Yeasts in the Presence of Anagen Hair Shaft Components.

4.1 SUMMARY

Malassezia yeasts are known to inhabit the pilosebaceous unit of a high percentage of healthy individuals and are closely associated with conditions known to affect hair follicles. The aim of this study was to ascertain if *Malassezia* species have a predilection for a specific component of the hair follicle.

Intact anagen hairs were plucked from the heads of healthy volunteers, microdissected into specific hair shaft components and infected with *Malassezia furfur in vitro* in PBS. Viability counts of yeast cells after three days incubation at 29°C in PBS cultures of *M. furfur* containing dissected scalp hair shaft components of newly washed hair from three out of five donors revealed stimulation of yeast cell growth by the nonemerged fully formed hair shaft compared to a PBS suspension control. Two out of five donors revealed stimulation of yeast cell growth by the emerged hair shaft. A similar pattern of stimulation was observed for *M. sympodialis* for three donors used in this study.

Isolated hair components were dissected and agitated in *M. furfur*-PBS suspensions for five days at 37°C. Scanning electron microscopy (SEM) was then used to assess adherence of *M. furfur* cells to follicle components. The emerged shaft samples on average had 2.98 ± 0.01 (sem, n=3) yeast cells per 50μm^2; the nonemerged fully formed shaft had 8.99 ± 0.18 (sem, n=3) yeast cells per 50μm^2; the

newly formed shaft stripped of the outer root sheath and inner root sheath had 0.26 ± 0.13 (sem, n=3) yeast cells per 50μm^2.

This experimental work has shown that *M. furfur* and *M. sympodialis* have a predilection for the lipid-rich areas of the hair shaft, in particular the nonemerged, fully keratinised shaft, though this may be affected by hair type.

4.2 INTRODUCTION

It is known that the pilosebaceous unit (PSU) can be colonised by saprophytic bacteria and provides a site of growth for the majority of saprophytic bacteria found on nonhydrated human skin [134]. The autochthonous resident microbial populations observed on the skin surface and in the PSU normally consists of organisms from the genera *Malassezia, Propionibacteria* and *Staphylococci*. The same organisms are also observed in the diseases dandruff and seborrhoeic dermatitis but in different proportions [151].

Various factors contributing to follicular colonisation are thought to include nutritional deficiency, high sebum turnover rate, low water activity and non-specific host antimicrobial activity [134]. Factors such as age, sex and acne severity have been shown not to influence colonisation of follicular ducts [134].

The main studies of scalp flora that have been carried out previously have usually been nonqualitative, direct cell counts often being employed. When such studies were found to provide insufficient, highly variable information as to the population densities of these organisms, other quantitative methods of analysis have been employed. These methods include techniques such as direct analysis of skin scrapings, D-squames biopsy [172]; punch biopsy [146];contact plates [20]; and the Williamson and Kligman scrub technique [238]. However, each technique has its drawback and the method utilised is chosen based on the experimental objective, the method feasibility and limitations.

Follicular colonisation patterns have also been notoriously difficult to assess. Sampling techniques used on human skin fail both in sampling the follicular environment efficiently and in differentiating the contents of individual follicles [134]. Kellum [122] devised a skin biopsy method for the isolation of intact PSUs which allowed the examination of PSU contents in isolation from neighbouring skin surface and follicular environments [122] and has allowed other investigators to assess the microbial colonisation of the PSU [135,179]. This procedure consisted of isolation of PSUs from skin biopsies after treatment with 1M-$CaCl_2$ solution to facilitate detachment of the epidermis and attached follicle from the dermis under a dissecting microscope. Each individual follicle could then be cut from the epidermis and homogenised individually in phosphate-buffered 0.1% (v/v) Triton X-100 solution. Viable counts could then be made on the follicular homogenates with diagnostic agar for each organism being used for viability plate counting. Puhvel *et al* [179] employed this technique to assess colonisation patterns of PSUs by bacteria in follicles isolated from normal skin. Leeming *et al* then confirmed these results and

employed the same technique to examine the ecology of normal PSUs and also of acne vulgaris comedones [134,135]. Although these techniques have not been used to assess follicular colonisation in seborrhoeic dermatitis, pityriasis versicolor dandruff or folliculitis, they could easily be extrapolated for use in assessing follicular colonisation in these disease states.

Some investigators have suggested that the stratum corneum is more frequently colonised than the pilosebaceous unit [148]. A study by Leeming *et al* [134] showed that *Pityrosporum (Malassezia)* species were the third major microbial group isolated from the hair follicle. The intrafollicular incidence of all colonising organisms was found to be lower than at the skin surface, but was thought to be adequate to explain the surface densities of *Pityrosporum*, *Propionibacteria* and *Staphylococcus* species, suggesting that the PSU is the primary growth site of these organisms [134]. The methods outlined by Kellum [122] and Leeming *et al* [135] have mostly been used to assess the role of colonising micro-organisms in acne vulgaris and the formation of comedones.

Quantitative studies of the changes in population size at the scalp surface have shown that *Pityrosporum* species made up 46% of the total microflora in normals, 74% in dandruff and 83% in seborrhoeic dermatitis [151]. McGinley *et al* [151] concluded that *Malassezia* were the only organisms significantly increased in dandruff. The population of *Propionibacteria* was also found to change between normals and dandruff patients. It is not fully established whether *Propionibacteria* contribute to *Malassezia*-related infections but it is possible that there may be

increased populations of *Propionibacteria* at the infundibular level in these disease states [151].

Transient colonisers of the scalp may include the generas *Klebsiella, Proteus* , *Bacillus* and *Candida* species. A study by Roia and Vanderwyk[192] identified 225 residential isolates including 30 yeasts, 143 moulds, 44 bacteria and 8 actinomycetes as resident organisms in both dandruff sufferers and normal patients. However, this study did not take into consideration transient colonisation from the environment. Because the hair provides a good trap for micro-organisms it is feasible that environmental organisms may be trapped from the surrounding environment and transiently reside on the scalp surface. In the presence of tinea infections, keratinophilic dermatophytes, such as *Trichophyton tonsurans* or *Microsporum canis* may also be resident. To date, these organisms are the only fungi observed to cause invasive diseases of human hair in an ectothrix or endothrix manner. By using a dissected hair shaft model it was possible to investigate such properties in *Malassezia* yeasts or highlight mechanisms involved in colonisation and growth.

4.3 AIM OF THE STUDY

Although there are now documented studies of the microbial ecology of the PSU, there have been no further studies which have taken into consideration the regions of the hair shaft or PSU colonised by micro-organisms or whether there are any stimulatory or inhibitory factors present in the follicle for microbial growth. In the

present study, plucked anagen hairs from the scalps of healthy volunteers have been used to assess growth, colonisation and invasion of the hair shaft components by *Malassezia* yeasts. The object of this study was to elucidate which follicular regions may be preferentially colonised by *Malassezia* yeasts and to assess whether invasion occurs.

4.4 METHODS

4.4.1 Isolates

A strain designated as "Hook" (*M. furfur*) was isolated from a scalp psoriasis study patient at the Western infirmary Glasgow, UK. Isolate GM340 (*M. sympodialis*), was kindly donated by Dr G Midgley, St John's Institute of Dermatology, London, UK. *M. pachydermatis* was kindly donated by Dr J Faergemann, Gothenburg, Sweden. D83640 (*Candida albicans*) was isolated from a fingernail sample at the Regional Mycology Reference Lab., Western Infirmary, Glasgow, UK. *Malassezia* isolates were grown for 24h in modified Dixon's broth at 29°C. *C. albicans* was grown in glucose-peptone broth for 24h at 29°C. In some instances, *Malassezia* isolates were grown in Dorn's filamentation medium [63] to achieve a dimorphic population.

Five ml aliquots of the fungal suspensions were centrifuged and washed three times in Dulbecco's PBS containing 1% absolute alcohol before agitation with glass beads to achieve a single cell suspension. Yeast cell counts were then carried out with a haemocytometer and subsequently diluted to achieve stock suspensions of approximately $1x10^8$ yeast cells ml^{-1}.

4.4.2 Donors

Donors with variable hair washing patterns were used to obtain hair shaft samples. Donor A washed their hair twice daily; Donor B, daily; Donor C, every 48h; Donor D, every 72h; Donor E, every 96h. Donors using any antidandruff shampoos or other medicated washing formulations were excluded.

4.4.3 Microdissection of hair shaft

Scalp anagen hair follicles were plucked from washed heads of healthy volunteers and microdissected into three main shaft components: the emerged shaft, which has penetrated the scalp surface; the nonemerged, fully keratinised hair shaft which lies directly above the sebaceous gland; and the newly formed (unsheathed shaft), obtained by mechanical stripping of the outer and inner root sheaths (Fig 4.1).

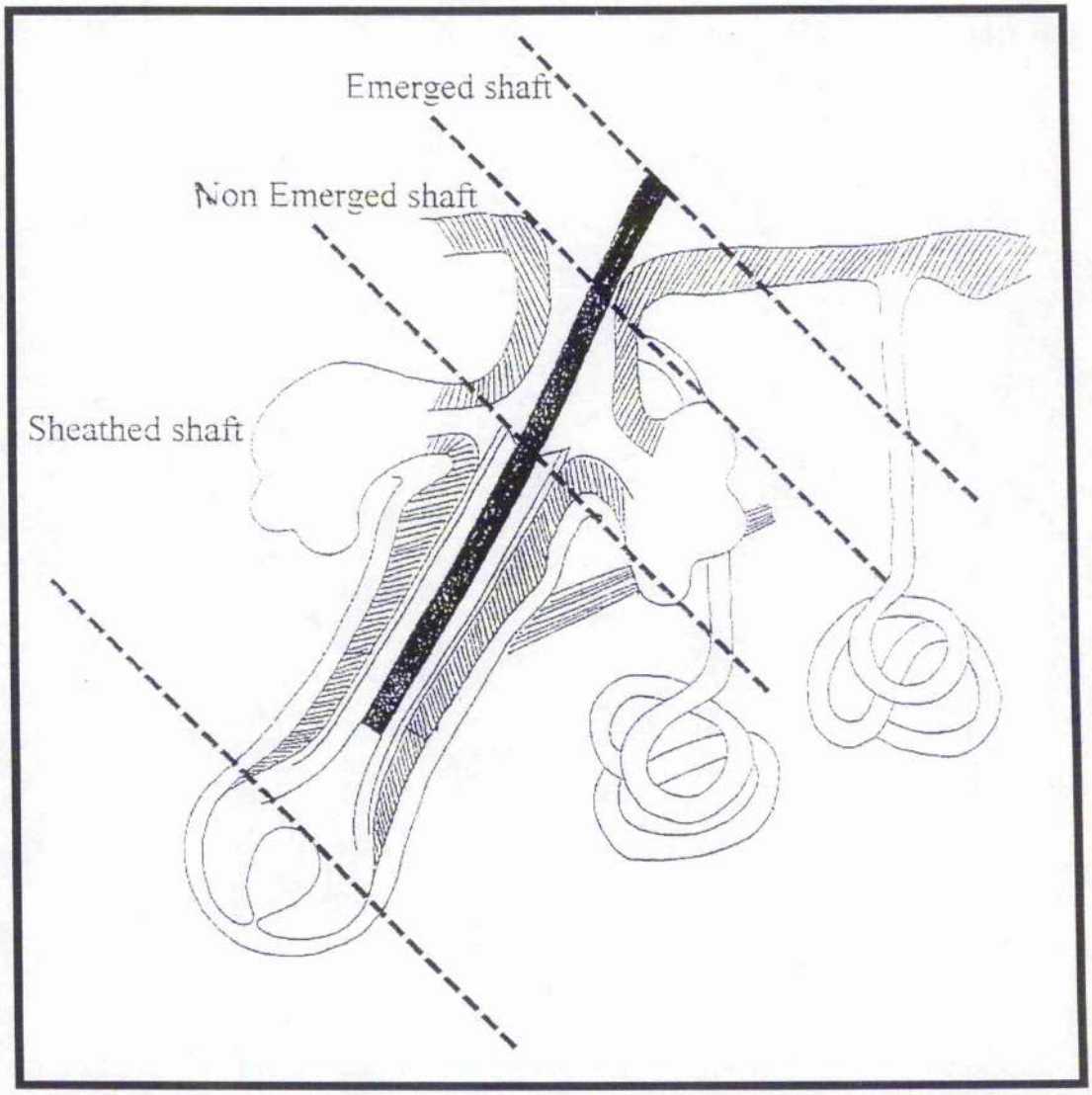

Figure 4.1. Diagram of the pilosebaceous unit. Represented are the three main shaft regions used in these experiments. These are the emerged shaft, which has penetrated the scalp surface; the nonemerged shaft which essentially lies in a pool of sebum, directly above the sebaceous gland; and the newly formed shaft encased in the inner root sheath and outer root sheath.

4.4.4 Suspension of hair shaft components on floating filters

Anagen hair shafts were plucked and microdissected into three main components. Four pieces of each specific component were then placed upon 13mm 5μm pore nucleopore membrane polycarbonate filters which were then floated on PBS in a 16 well dish. Each filter was then inoculated with $1x10^8$ yeast cell ml^{-1} and incubated at 37^oC for 3 days. Samples could then be examined by confocal microscopy or scanning electron microscopy (SEM).

4.4.5 Examination of infected hair shaft components by confocal laser scanning microscopy.

Infected hair shaft samples were set up as in 4.4.3. After incubation, the samples and a control, uninoculated hair shaft were then stained with an anti-*P. ovale* polyclonal antibody which was tested for specificity to the isolate being examined. For fluorescent immunostaining, the samples were blocked with a 1/20 normal swine serum solution for 15 min before addition of an anti-*P. ovale* polyclonal raised from rabbit at a 1/40 concentration. After washing in Tris saline (TBS) SAPU FITC antirabbit (donkey) conjugate (1/50) was added for 30 min. Samples were then washed in TBS before mounting in 60% glycerine in methylated spirits for

observation with the confocal microscope. The anti-*P. ovale* antibody was kindly donated by Dr HR Ashbee, Dept. Microbiology, University of Leeds.

4.4.6 Quantification of colonisation of follicular regions by *M. furfur*

Dissected hair shaft components were added to 100 µL suspensions of *M. furfur* in Dulbecco's PBS. Five pieces of each component were added to the respective cultures. Incubation was carried out at 29°C for five days with agitation to ensure a constant yeast cell suspension. After incubation, hair components were removed, washed once in PBS and fixed and processed for SEM. Once the samples had been prepared they were visually examined using SEM to quantify the number of adherent yeast cells. This was achieved by photographing each sample at a constant magnification of x1500. Once the micrographs were developed, a 50µm^2 transparent grid proportionate to the 10µm bar measurement on the micrographs was constructed, which could be superimposed upon each micrograph. The number of yeast cells per 10µm^2 were counted over 100 fields of view (1 field of view = 10µm^2).

4.4.7 Scanning electron microscopy (SEM)

After incubation, samples were fixed in 2.5% glutaraldehyde in 1.0M Sorrenson's buffer, pH 7.4 for 1h. After washing in buffer for 45min the hair components were

postfixed in 1% osmium tetroxide and rinsed again in buffer followed by successive dehydration steps in graded ethanol solutions (25-100%), air dried for 24h, mounted on 10mm aluminium stubs, sputter coated with gold and examined in a JSM 6400 scanning electron microscope.

4.4.8 Growth of *M. furfur* and *M. sympodialis* in the presence of hairshaft components

Five pieces of each hairshaft component were added to 100µl suspensions *of M. furfur* or *M. sympodialis* in PBS, containing approximately $1x10^7$ yeast cells ml^{-1}. Samples were then incubated for 72h at 29°C with constant agitation to ensure an even yeast cell suspension. At 0h and 72h, viability counts were carried out by plating out 100µl aliquots of serial dilutions (1:10) of 10µl aliquots of the samples onto modified Dixon's agar and incubating for three days at 37°C after which time the number of viable colonies at each time point was counted and subsequently growth could be assessed by the differences in number of viable colonies at each time point. This procedure was carried out for a variety of donors with different hair washing regimes.

4.4.9 Growth of *M. furfur* and *M. sympodialis* in hairshaft component-conditioned PBS.

Five pieces of each follicular shaft component were added to 90µl aliquots of Dulbecco's PBS and incubated at 29°C for two days after which time the components were removed. Ten microlitre suspensions of *M. furfur* or *M. sympodialis* in PBS were then added to the "conditioned" PBS samples to achieve a final concentration of $1x10^7$yeast cells $ml^{-1}$and cultures were then incubated at 29°C for 96h. At 0h (post-conditioning) and 96h, growth *of M. furfur* was assessed by the method outlined in 4.4.8.

4.4.10 Statistical analyses

Conventional methods of statistical analyses were not appropriate for analyses of these experimental results, due to the many sources of variation and the lack of enough donors/samples tested for each set of experiments. It was thought that to generate enough samples to limit all sources of variation would be time consuming and impractical. However, it was still appropriate to gain an overall feel for the result trends generated in this study. To determine whether the increase in growth observed per shaft component was significant and whether the differences in increase between datasets was significant, the data was manipulated by comparing the means and ranges of groups of replicates and donors for each time point. Once the minimum differences between averages and ranges were calculated for each dataset, the final comparisons were made by averaging each zero hour dataset separately , averaging all 72 hour results together and expressing each zero hour average as a proportion of the total 72 hour average. These figures enabled the differences between results to be

observed clearly to give an overall trend for the data. These results are given in Table 4.5 and Table 4.6.

4.5 RESULTS

4.5.1 Examination of infected hair shaft components by confocal microscopy

For all samples and controls examined, yeast cells (added and endogenous) appeared to have a predilection for the nonemerged shaft (Figure 4.2, Figure 4.3). A control specimen revealed few fluorescent bodies, reinforcing the positive immunostaining of *M. furfur*. Fluorescent particles observed in control specimen may be due to staining of endogenous yeast cells (Figure 4.4). Due to the method of inoculation these results cannot be used quantitatively, but may give a primary indication of a predilection for colonisation of the nonemerged shaft.

4.5.2 Scanning electron microscopy of floating filter samples.

On examination, fibrin-like structures from yeast-to-yeast were observed (Figure 4.5). Clumps of yeast cells were found at random intervals along the intact specimen with variable morphology and bud elongation being observed (Figure 4.6). An extracellular coating was found over some clumps of yeast cells (Figure 4.7). Much cuticular destruction was observed in some samples (Figure 4.8) which was not readily observed in control specimens (Figure 4.9). When a dimorphic inoculum was used, short filaments were observed to extend along cuticular surfaces or be associated with clumps of yeast cells (Figure 4.10), with no evidence of penetration beneath the cuticular layer (Figure 4.11). On nonemerged shaft-infected samples, yeast cells were observed to be associated with follicular debris and deposits of unknown origin (Figure 4.12). These deposits were not observed on stripped shaft or in rare cases on emerged shaft specimens (Figure 4.13). The observations made on yeast cell morphology were apparent at all regions of the hair shaft examined, and are summarised in Table 4.1.

4.5.3 Quantification of colonisation of follicular regions by *M. furfur*

In three independent experiments, it appeared that the nonemerged shaft was the most colonised region of the hairshaft (Table 4.2). Non-parametric analyses of the collected data showed that the colonisation observed at this region was highly significant. Colonisation of the emerged shaft and unsheathed shaft was found to be nonsignificant. Although yeast cells were observed on the emerged shaft and unsheathed shaft in section 4.5.2, the method of inoculation of the floating filter

samples did not ensure uniform distribution of the inocula over the entire shaft regions being examined. The method used in this section limited the variation in the amount of inoculum being distributed to each hair shaft region, hence the colonisation patterns here represent a more accurate reflection of hair shaft regions that are colonised. The observations made are summarised in Table 4.3.

4.5.4 Morphological observations from dissected hair shafts incubated in *M. furfur*/PBS suspensions.

The yeast morphology appeared to be variable at all regions of the follicle. On the emerged and nonemerged shaft, elongating buds and oval yeast cells were frequently observed. In some cases, yeast cells were observed to be covered in a surface coating and appeared to be associated with bacillus bacteria or aerobic cocci, particularly on the nonemerged region. On 4/5 nonemerged shaft samples analysed, *M. furfur* could be observed associated with follicular debris and deposits of unknown origin, possibly sebum (Figure 4.14). It was also observed that when the cuticular layer of the hair was destroyed, yeast cells colonised cortical tissue. Again morphology was variable and fungal filaments could be observed penetrating cortical tissue (Figure 4.15). In a similar experiment where hair shaft components were added to a liquid suspension containing yeast cells and filaments, the filaments were not observed to penetrate the cuticular layer but were observed to extend along the follicular surfaces (Figure 4.16).

4.5.5 Growth of *M. furfur* and *M. sympodialis* in the presence of follicular shaft components

M. furfur growth in the presence of hair components was variable between different donors. Hairshaft components from donors who washed their hair at least once to twice daily did not stimulate *M.furfur* growth by 96h. A decrease in viable yeast cell growth with time for each shaft component was observed for donor A (Figure 4.17). Decreases in viable yeast cell growth for donor B (Figure 4.18) were also observed. In cultures containing shaft components from donor C, an increase in growth was observed in cultures containing nonemerged shaft components only (Figure 4.19). Donor D also showed a 2.3-fold increase in viable yeast cell growth in cultures containing nonemerged hair shaft components, as well as an increase in cultures containing emerged shaft components (Figure 4.20). When hair shaft components were used from donor E who normally left 96h between hair washes, increases in growth was observed in emerged shaft cultures, nonemerged shaft cultures and unsheathed shaft cultures (Figure 4.21). Significant changes in growth were observed for donor C when their hair was left unwashed for 5d. Increases in growth were observed in emerged shaft cultures , nonemerged shaft cultures and unsheathed shaft cultures (Figure 4.22). Viable growth of *M. sympodialis* in the presence of hair shaft components from donors B, C and E was also assessed. For donor B, no increase in growth was observed (Figure 4.23). For donor C, an increase in growth was observed in nonemerged shaft cultures alone (Figure 4.24) and for donor E in emerged shaft cultures, nonemerged shaft cultures and unsheathed shaft cultures (Figure 4.25),

which reflect the results observed for *M. furfur*. For donor C, the effects of hairshaft components on viable growth of *M. pachydermatis* and *C.albicans* was also assessed. In both cases, no viable growth was observed in any of the cultures examined (Figure 4.26, Figure 4.27).The results are summarised in Table 4.4. Statistical analysis is summarised in Table 4.6.

4.5.6 Growth of *M. furfur* and *M. sympodialis* in hair shaft component conditioned PBS

PBS conditioned with hair components from donors C and D stimulated yeast cell growth in *M. furfur* cultures . For Donor D, growth stimulation was observed in all conditioned cultures examined (Figure 4.28). An increase in growth for cultures conditioned with hair components from donor C was also observed (Figure 4.29). When *M. sympodialis* was grown in conditioned PBS (donor D) growth was also observed over 96h (Figure 4.30). In comparison, when *C. albicans* was grown in Donor C-conditioned PBS cultures, no significant increase in viable yeast cells was observed (Figure 4.31).

The results are summarised in Table 4.5. Statistical analysis is summarised in Table 4.7.

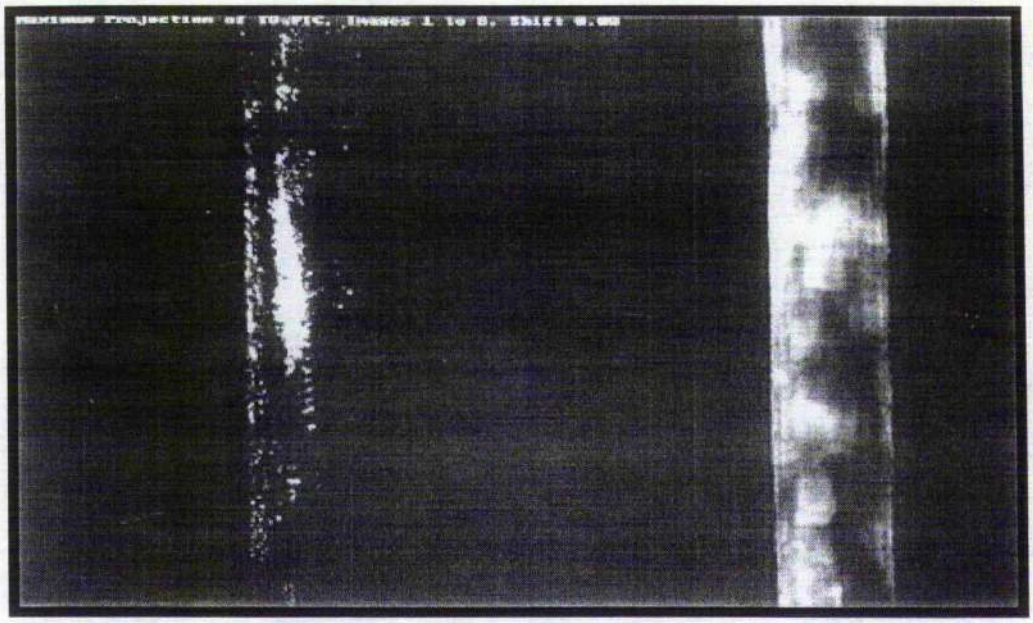

Figure 4.2. Fluorescent staining of *M. furfur* cells on nonemerged shaft component. This hair shaft region showed the greatest abundance of fluorescence compared to other shaft regions.

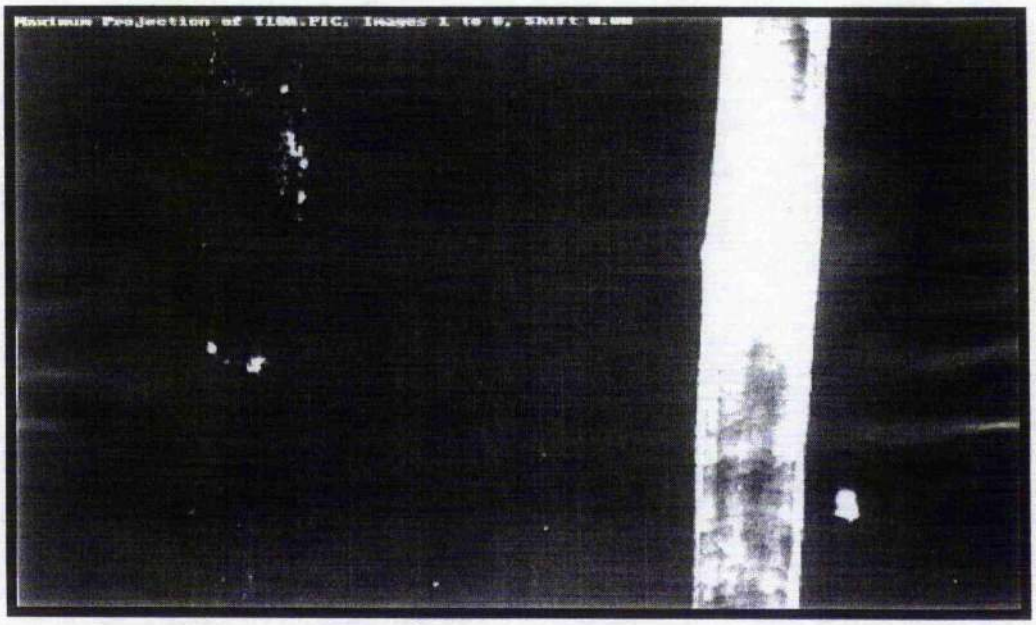

Figure 4.3. Fluorescent staining of endogenous particles on nonemerged shaft control sample. Although this sample was not inoculated, the fluorescent staining particles may be indicative of endogenous *Malassezia* colonisation.

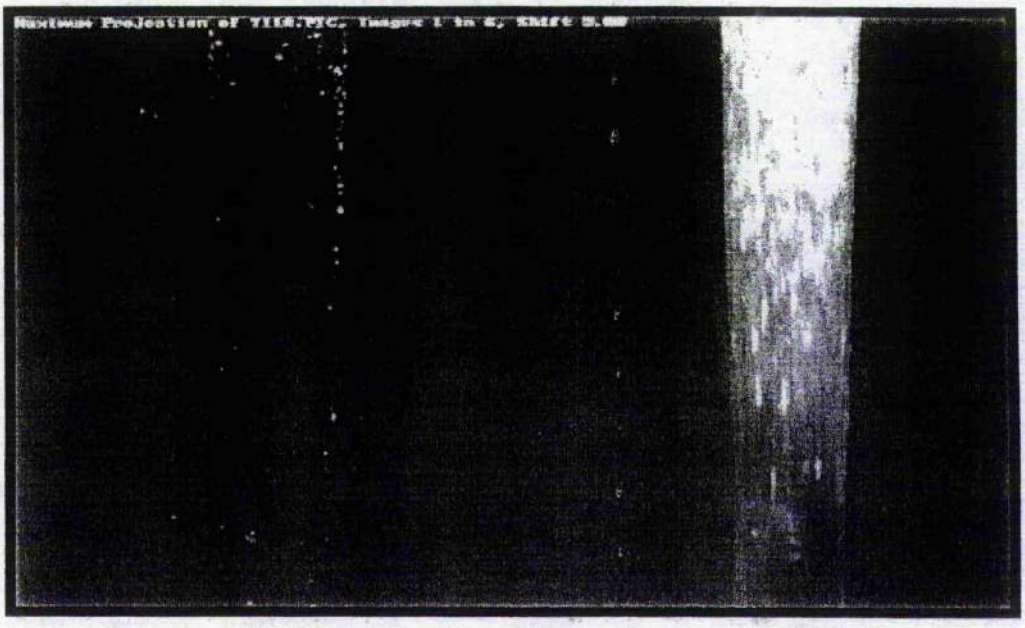

Figure 4.4. Fluorescently stained yeast cells on stripped hair shaft sample. These fluorescent bodies are much more infrequent than observed on nonemerged shaft samples.

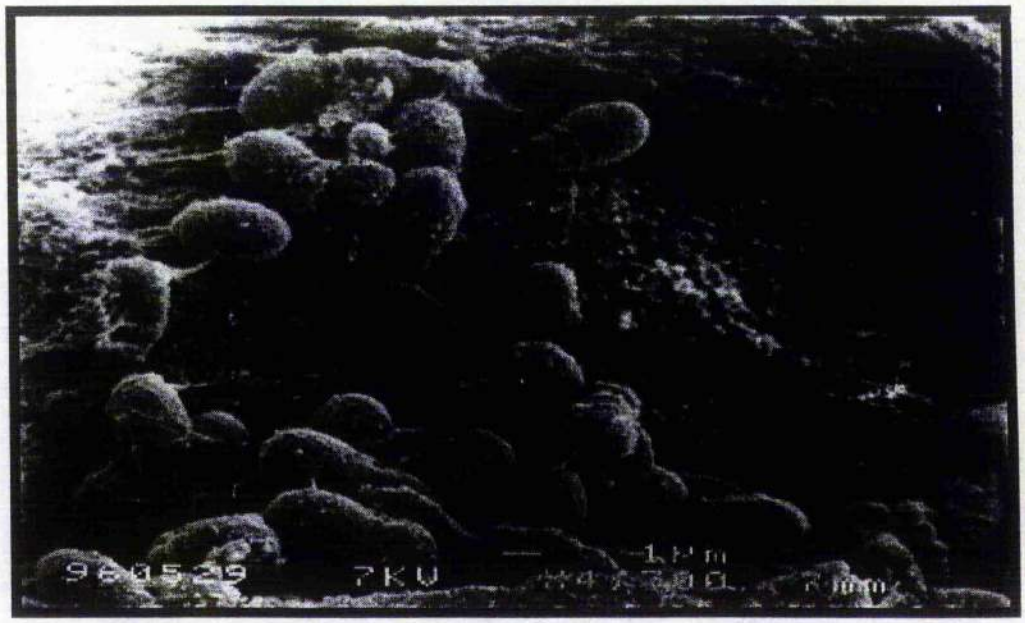

Figure 4.5. Fibre-like cell-to-cell structures between *M. furfur* yeast cells on the unsheathed region of hair shaft. These structures were abundantly present and were observed associated with clumps of yeast cells, attached to the hair shaft with a random distribution (X4, 300).

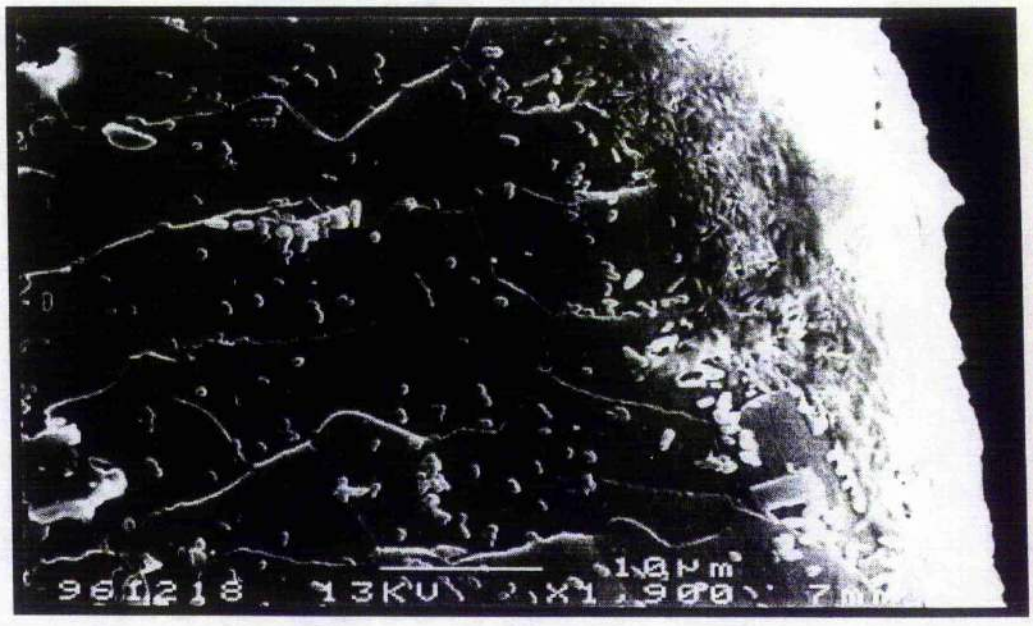

Figure 4.6. At all regions of the hair shaft, yeast cells showed evidence of over and under distribution. Large masses of yeast cells were observed as well as small clusters and single cells. Note the extracellular observed previously (X 1,900).

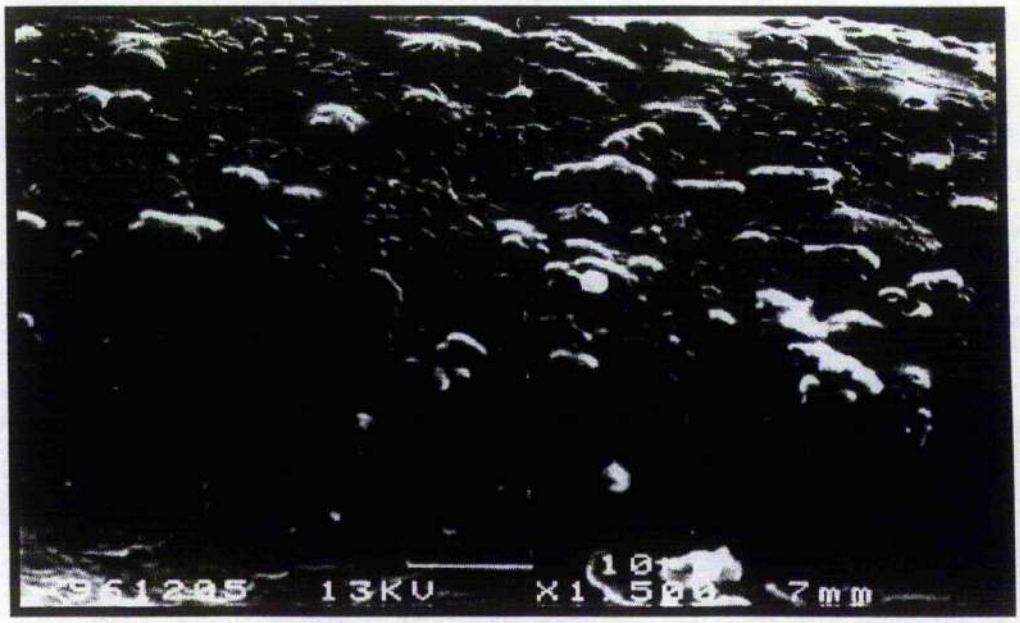

Figure 4.7. Extracellular coating associated with random clumps of yeast cells. Bacteria could also be observed in association with this coating either embedded or covered in this matrix (X1500).

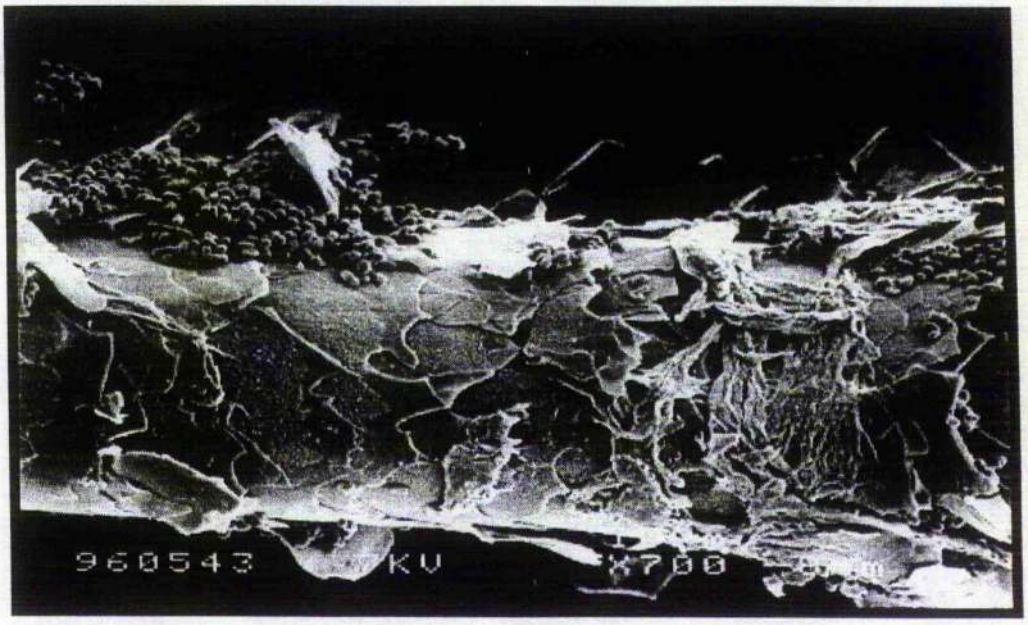

Figure 4.8. Cuticular destruction of the emerged shaft in the presence of *M. furfur*. Large masses of yeast cells appeared to be associated with areas of cuticular disruption and uplift. Where cuticles were raised, yeast cells were observed between the cuticular layers. In other cases, yeast cells were observed to be associated with flattened, intact cuticular surfaces (X 700).

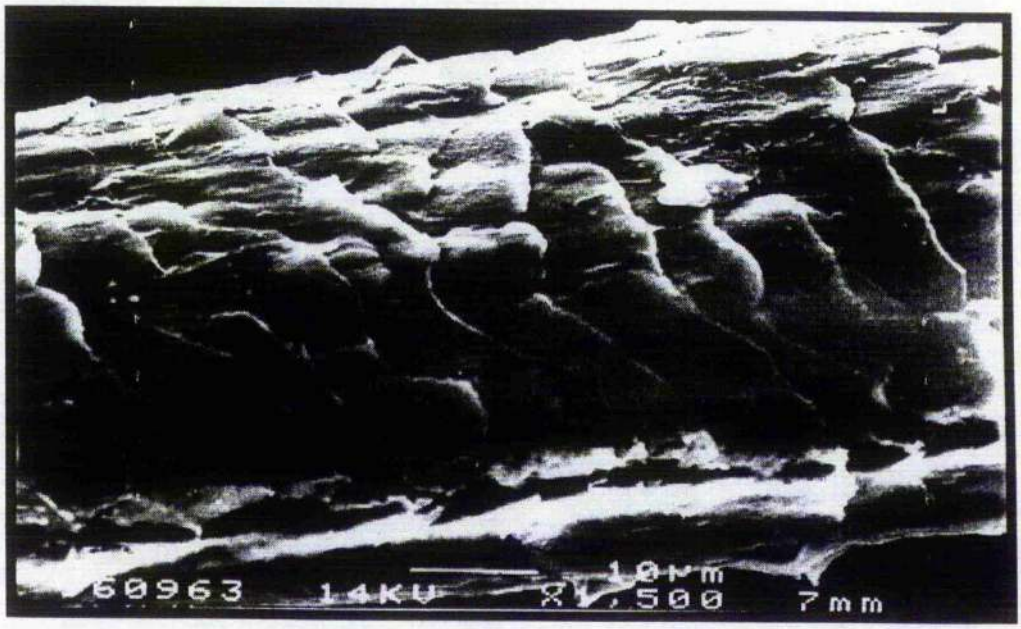

Figure 4.9. Micrograph showing a representative area of uninoculated hair shaft, incubated in the absence of *M. furfur* as a control for 4.9. Cuticles are observed intact and flattened compared to those observed in Figure 4.9. No evidence of any of the observations made in infected samples (Figures 4.6 to 4.9). (X1,500).

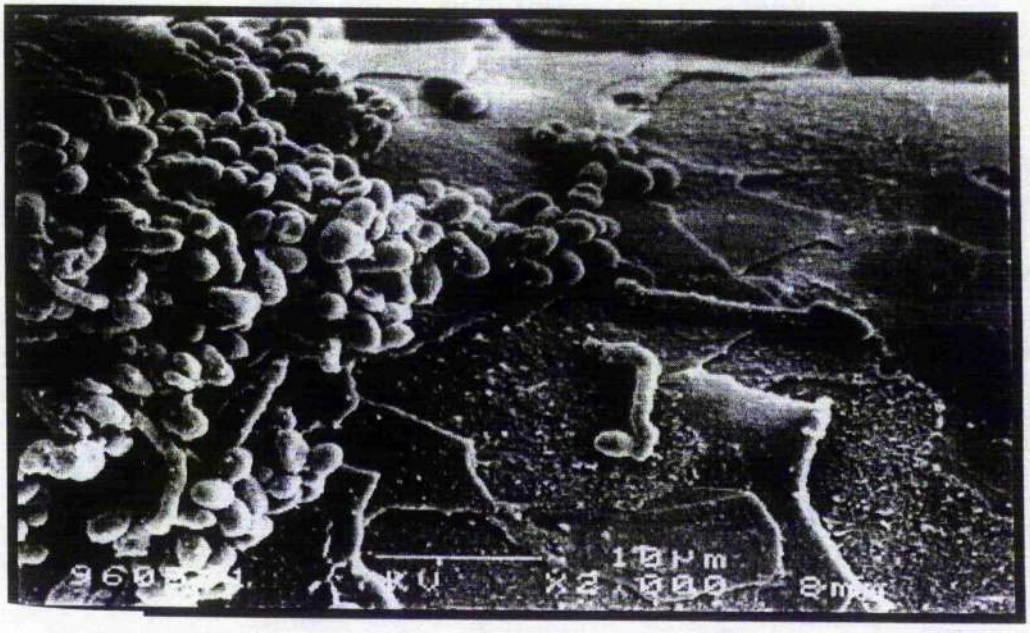

Figure 4.10. Fungal filaments were observed infrequently. Filaments were observed to extend along cuticular surfaces with no evidence of invasion. Clumps of yeast cells were also observed in association with flat cuticular surfaces (X2,000).

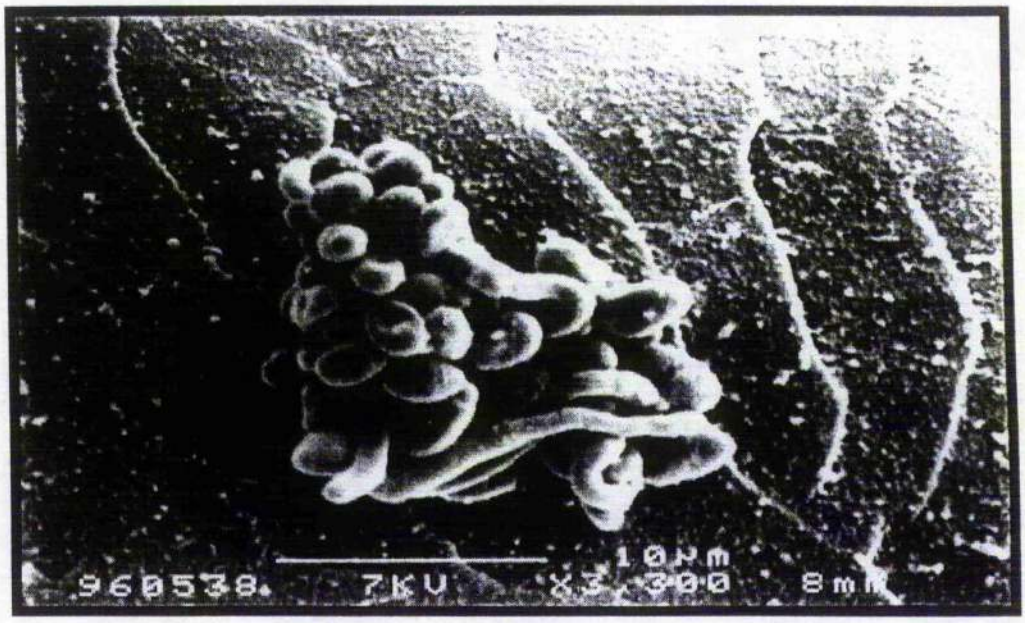

Figure 4.11. Fungal filaments were also observed associated with clumps of yeast cells on the cuticular layer of the hair shaft (X3,300).

Figure 4.12. On the nonemerged shaft region, yeast cells could be observed in association with follicular debris and deposits of unknown origin. Cocci bacteria were also observed at this location of the hair shaft (X1,500).

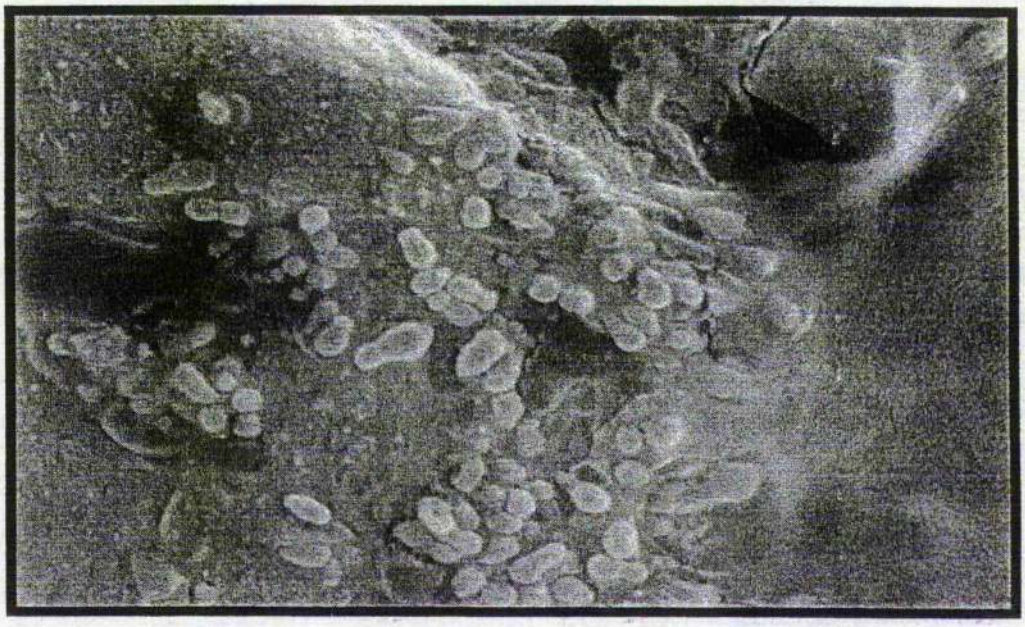

Figure 4.13. *M. furfur* yeast cells colonising the unsheathed shaft. Variable yeast cell morphology is observed with yeast cells embedding in a coating on the shaft surface. No evidence of follicular debris or other deposits (x3000).

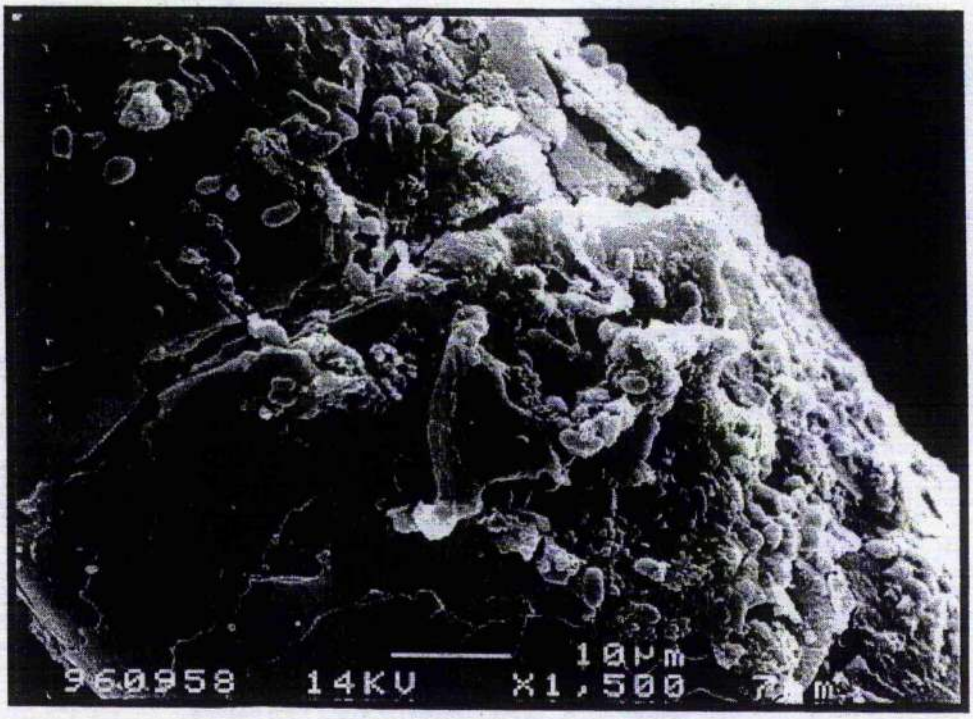

Figure 4.14. *M. furfur* in association with follicular debris and cuticular destruction on nonemerged shaft sample (x1500).

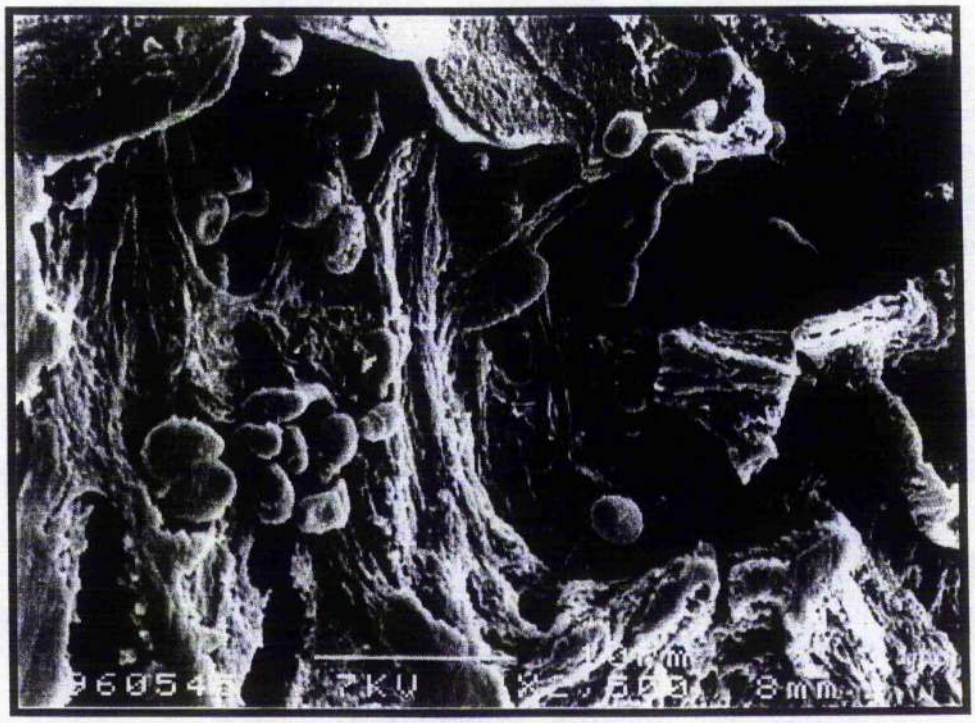

Figure 4.15. Penetration of cortical tissue by *M. furfur* when destruction to the cuticular layer has occurred . Variable morphologies are observed, x2,500.

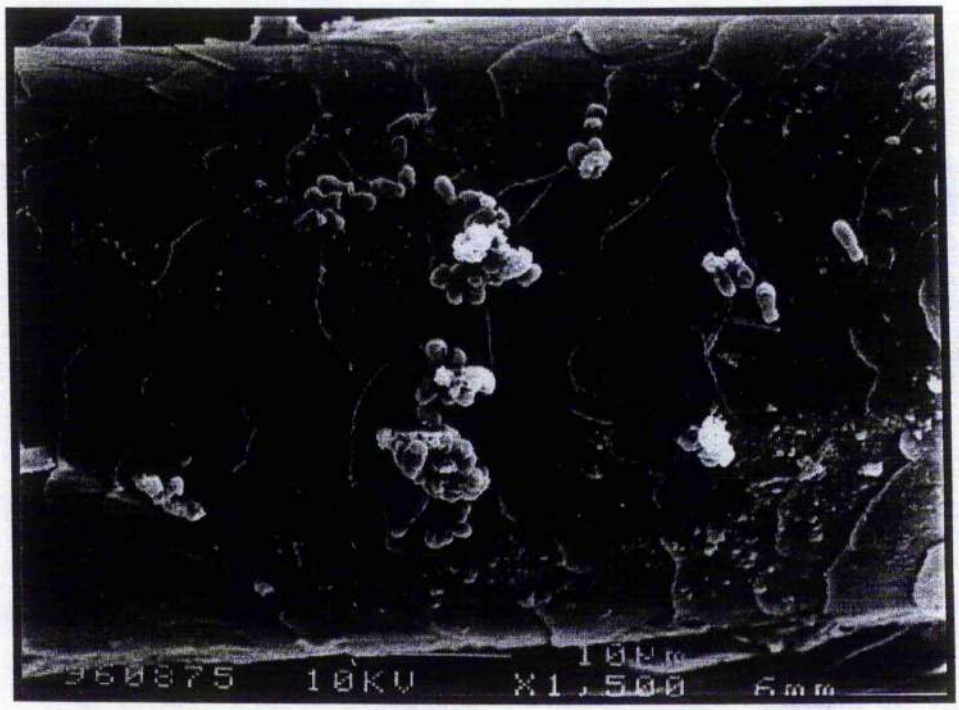

Figure 4.16. When a filamentous inoculum of *M. furfur* was added to hair shafts, the filaments were not observed to penetrate beneath the cuticular layer, but were observed in association with yeast cells to extend along cuticular surfaces, x1,500.

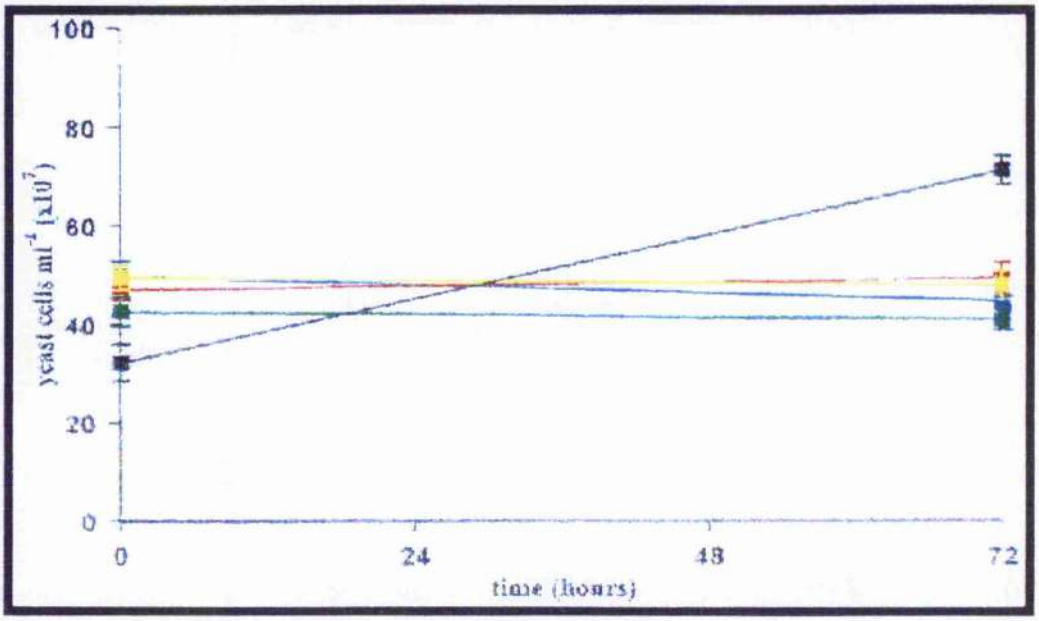

Figure 4.17. The effect of dissected shaft components from donor A on growth of *M. furfur*. No increase in viable number of yeast cells was observed in the presence of shaft components after 72 hours at 29°C. Dorn's medium control ■ PBS control ■ Emerged shaft ■ Nonemerged shaft ■ Unsheathed shaft ■.

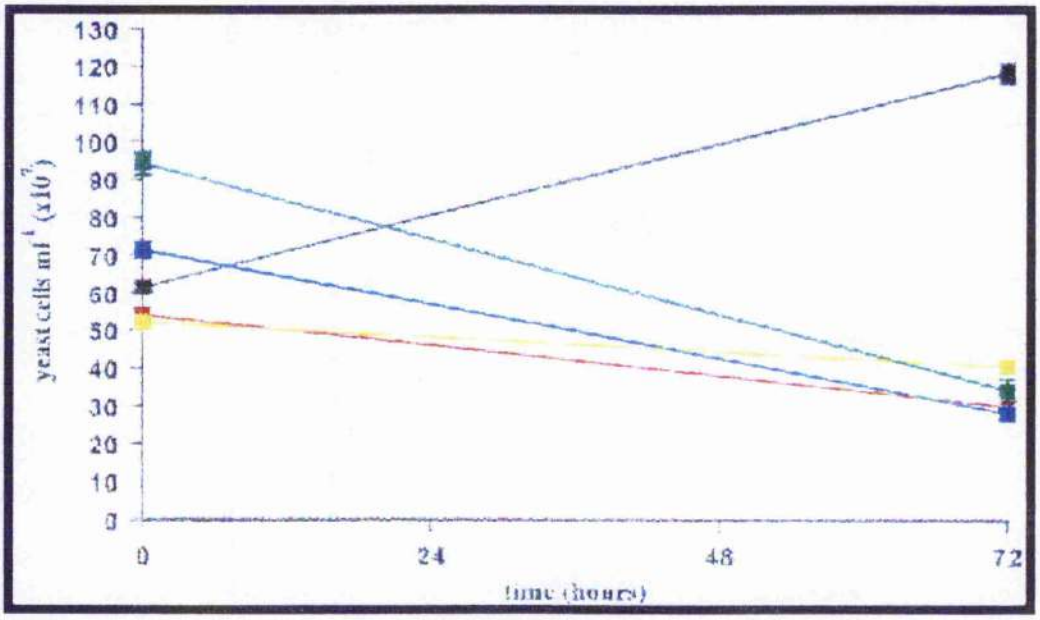

Figure 4.18. The effect of dissected shaft components from donor B on growth of *M. furfur*. No increase in viable number of yeast cells was observed in the presence of shaft components after 72 hours at 29°C. Dorn's medium control ■ PBS control ■ Emerged shaft ■ Nonemerged shaft ■ Unsheathed shaft ■.

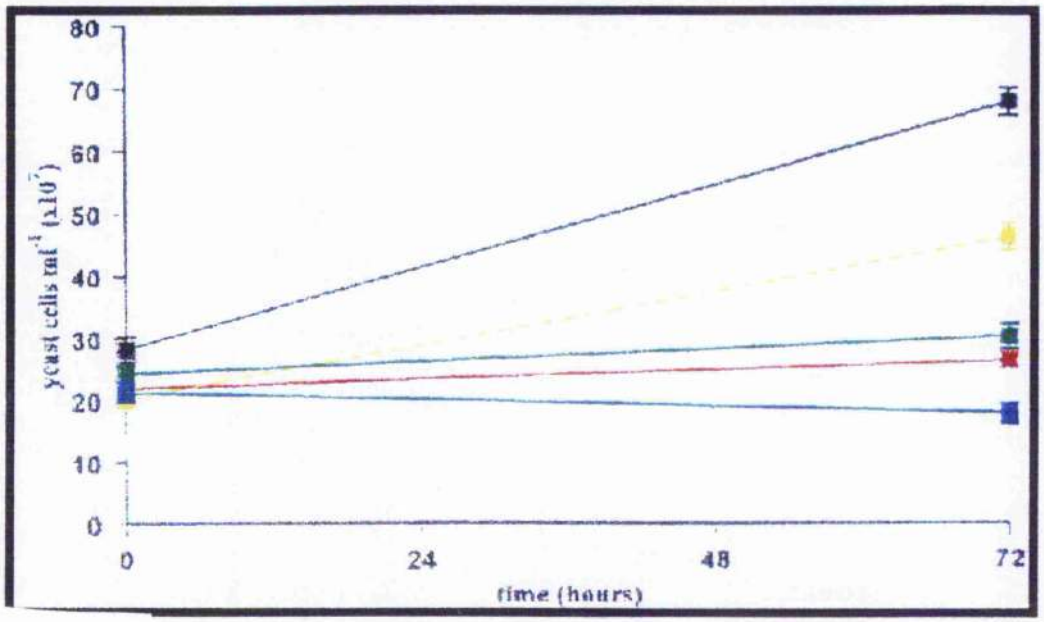

Figure 4.19. The effect of dissected shaft components from donor C on growth of *M. furfur*. An increase in viable number of yeast cells was observed in the presence of unemerged shaft components after 72 hours at 29°C. Dorn's medium control ■ PBS control ■ Emerged shaft ■ Nonemerged shaft ■ Unsheathed shaft ■.

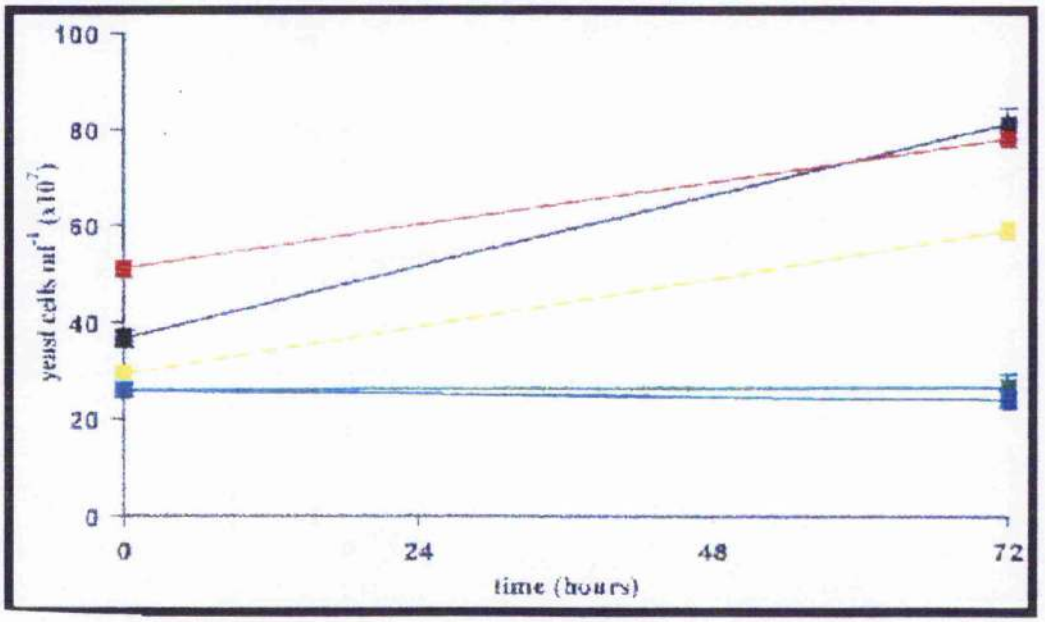

Figure 4.20 The effect of dissected shaft components from donor D on growth of *M. furfur*. An increase in viable number of yeast cells was observed in the presence of emerged shaft and nonemerged shaft components after 72 hours at 29°C. Dorn's medium control ■ PBS control ■ Emerged shaft ■ Nonemerged shaft ■ Unsheathed shaft ■.

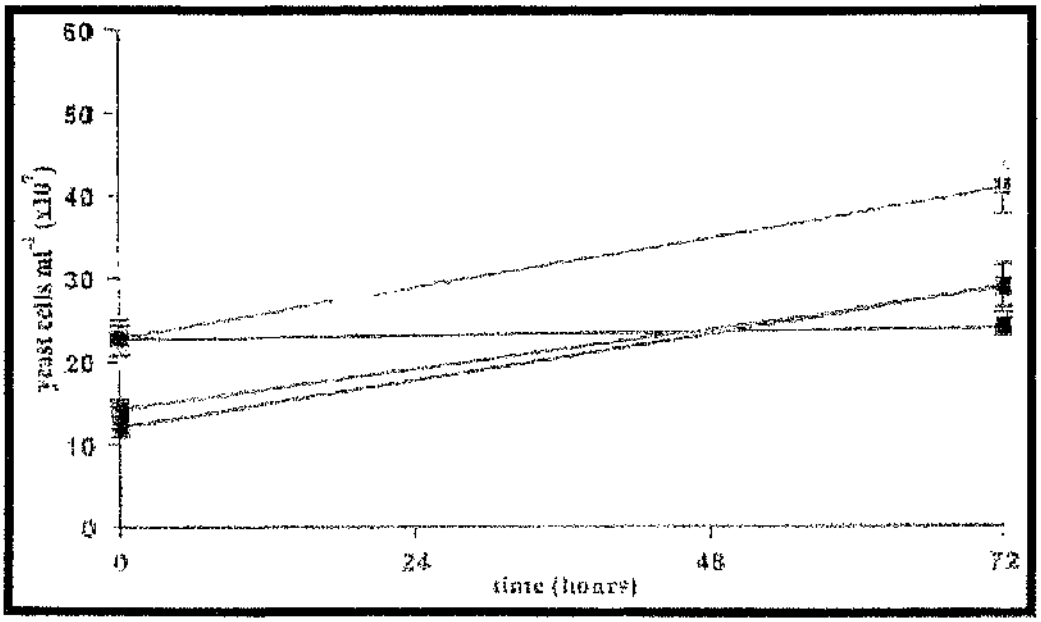

Figure 4.21. The effect of dissected shaft components from donor E on growth of *M. furfur*. An increase in viable number of yeast cells was observed in the presence of emerged shaft, unemerged shaft and unsheathed shaft components after 72 hours at 29°C. Dorn's medium control ■ PBS control ▨ Emerged shaft ▩ Nonemerged shaft ▩ Unsheathed shaft ▩.

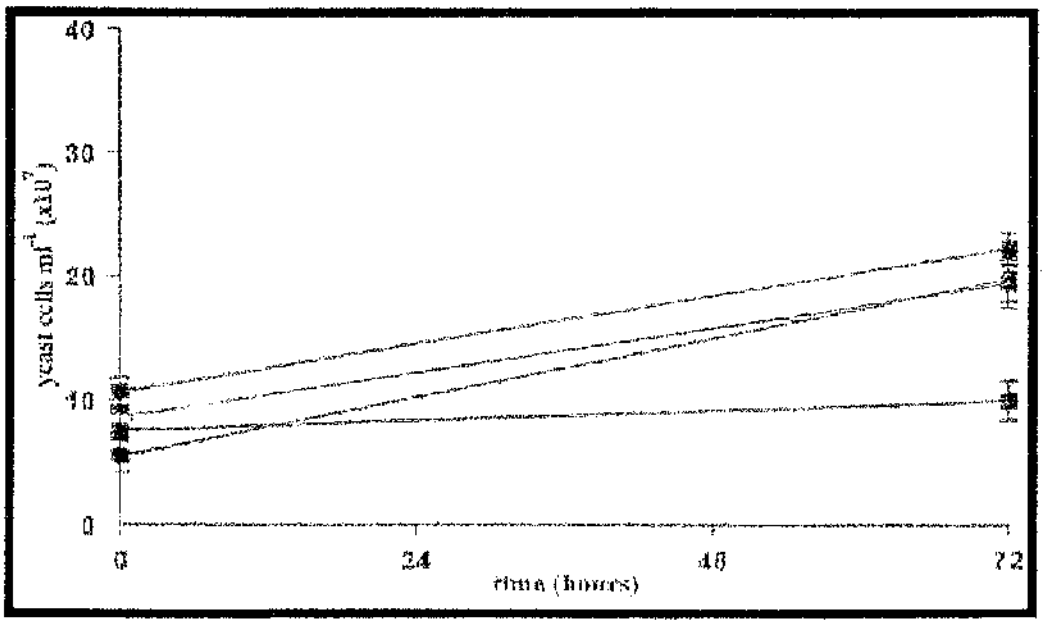

Figure 4.22. The effect of dissected shaft components from unwashed head (>5 days) of donor C on growth of *M. furfur*. An increase in viable number of yeast cells was observed in the presence of emerged shaft, unemerged shaft and unsheathed shaft components after 72 hours at 29°C. Dorn's medium control ■ PBS control ▨ Emerged shaft ▩ Nonemerged shaft ▩ Unsheathed shaft ▩.

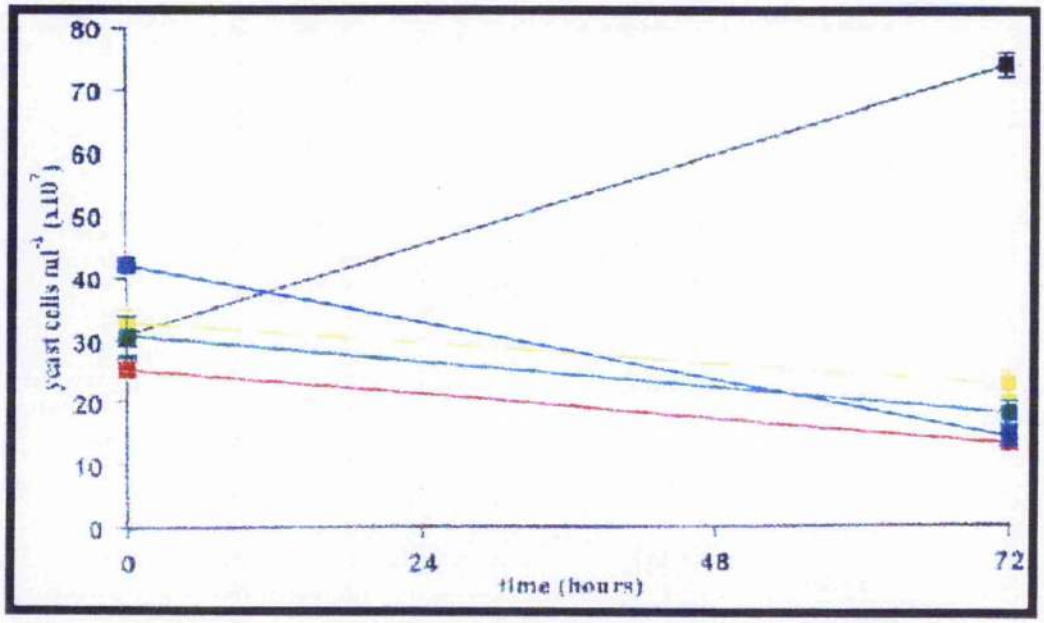

Figure 4.23. The effect of dissected shaft components from donor B on growth of *M. sympodialis*. An increase in viable number of yeast cells was observed only in the Dorn's medium positive control after 72 hours at 29°C. Dorn's medium control ■ PBS control ■ Emerged shaft ■ Nonemerged shaft ■ Unsheathed shaft ■.

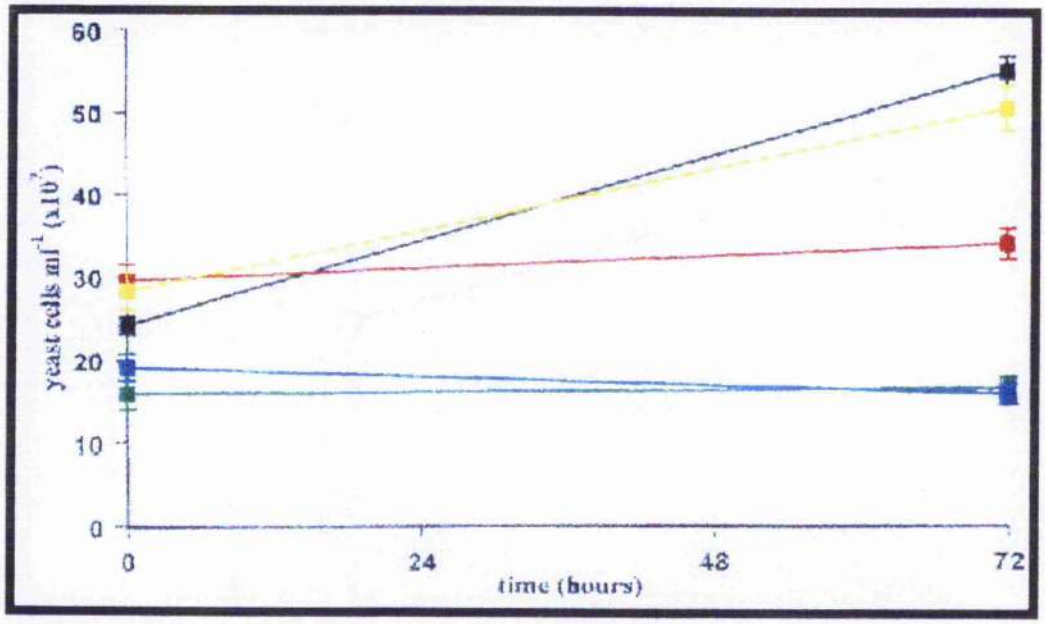

Figure 4.24. The effect of dissected shaft components from donor C on growth of *M. sympodialis*. An increase in viable number of yeast cells was observed in cultures containing nonemerged shaft components after 72 hours at 29°C. Dorn's medium control ■ PBS control ■ Emerged shaft ■ Nonemerged shaft ■ Unsheathed shaft ■.

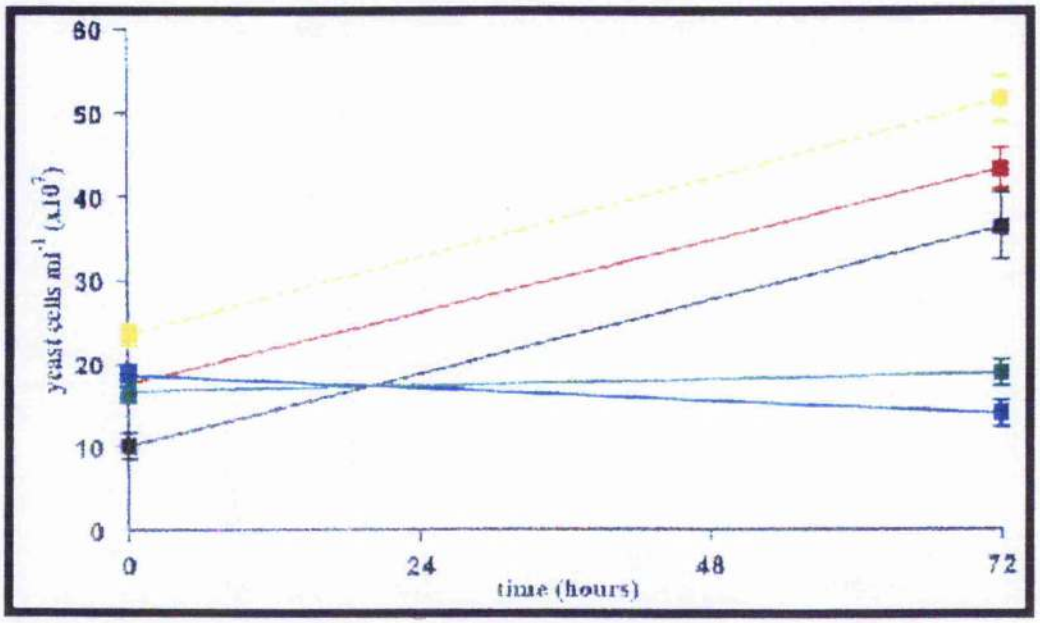

Figure 4.~~29~~ 25 The effect of dissected shaft components from donor E on growth of *M. sympodialis*. An increase in viable number of yeast cells was observed in cultures containing emerged shaft, nonemerged shaft and unsheathed shaft components after 72 hours at 29°C. Dorn's medium control ■ PBS control ■ Emerged shaft ■ Nonemerged shaft ■ Unsheathed shaft ■.

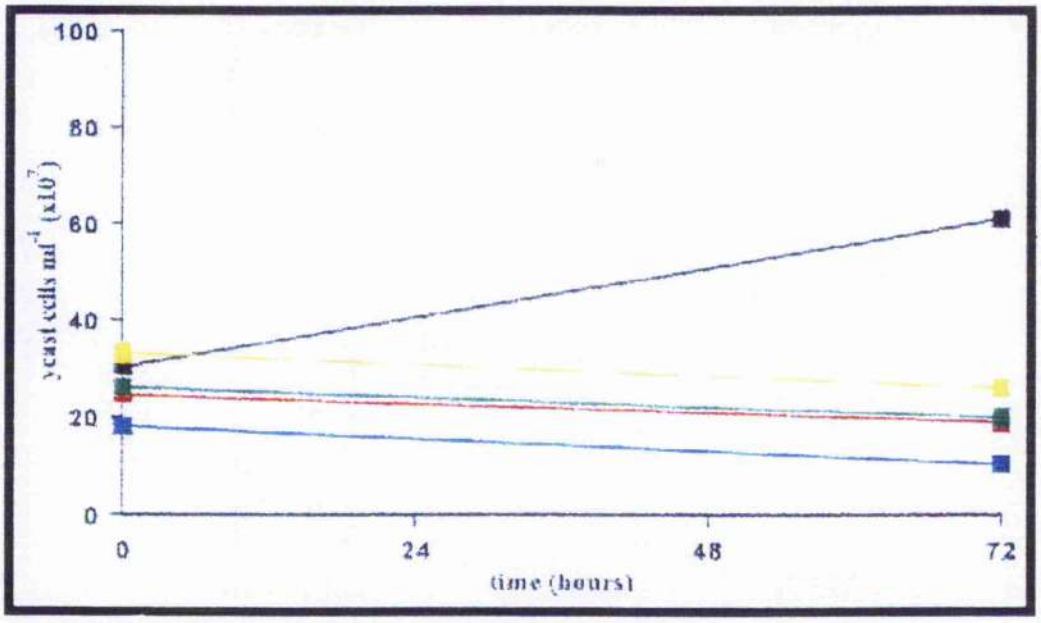

Figure 4.~~30~~ 26 The effect of dissected shaft components from donor C on growth of *M. pachydermatis*. No increase in viable number of yeast cells was observed in cultures hair shaft components after 72 hours at 29°C. Dorn's medium control ■ PBS control ■ Emerged shaft ■ Nonemerged shaft ■ Unsheathed shaft ■.

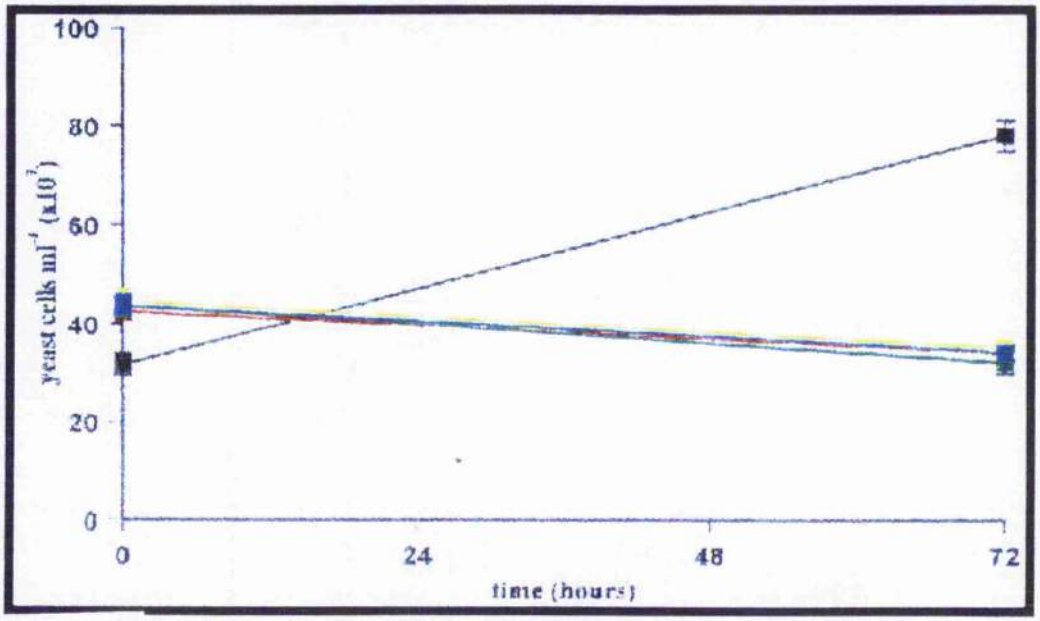

Figure 4.27. The effect of dissected shaft components from donor C on growth of *C. albicans*. No increase in viable number of yeast cells was observed in cultures containing hair shaft components after 72 hours at 29°C. Dorn's medium control ■ PBS control ■ Emerged shaft ■ Nonemerged shaft ■ Unsheathed shaft ■.

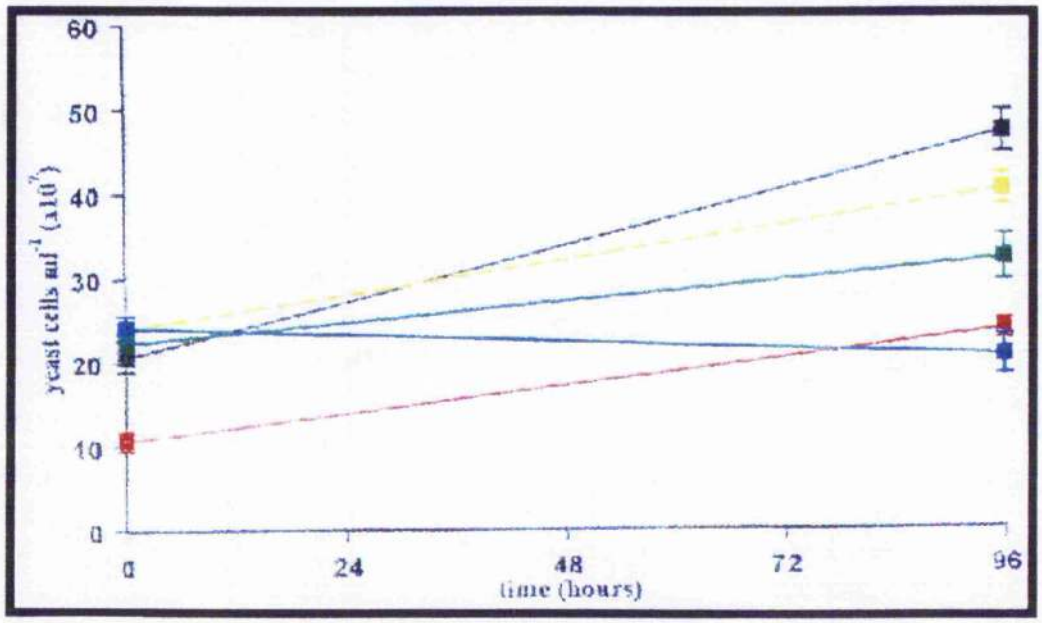

Figure 4.28. Growth of *M. furfur* in follicle component-conditioned PBS. When hair shaft components from donor D were used to condition PBS for 48 hours before the addition of the inoculum, an increase in colony formimg units was observed over 96h at 29°C for all conditioned cultures. Dorn's medium control ■ PBS control ■ Emerged shaft ■ Nonemerged shaft ■ Unsheathed shaft ■.

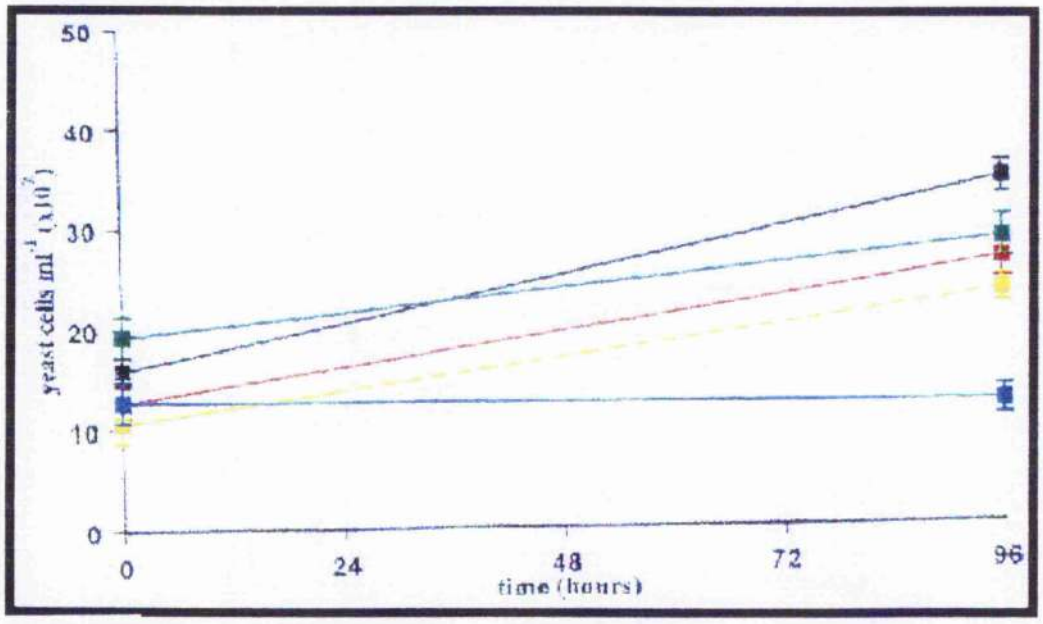

Figure 4.29. Growth of *M. furfur* in follicle component-conditioned PBS. When hair shaft components from donor C were used to condition PBS for 48 hours before the addition of the inoculum, an increase in colony formimg units was observed over 96h at 29°C for all conditioned cultures. Dorn's medium control ■ PBS control ■ Emerged shaft ■ Nonemerged shaft ■ Unsheathed shaft ■.

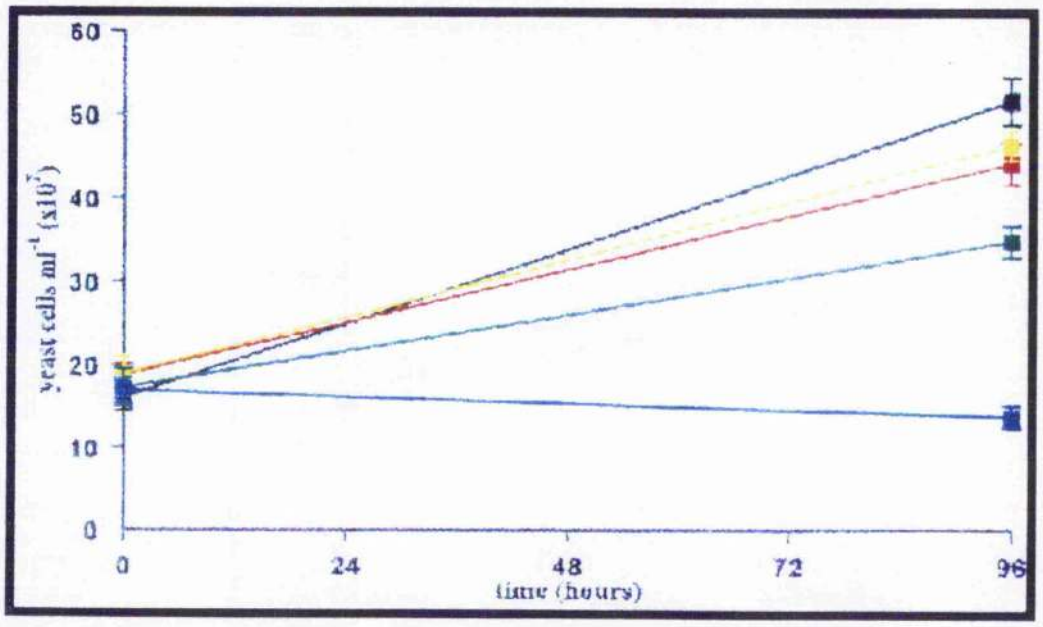

Figure 4.30. Growth of *M. sympodialis* in follicle component-conditioned PBS. When hair shaft components from donor D were used to condition PBS for 48 hours before the addition of the inoculum, an increase in colony forming units was observed over 96h at 29°C for all conditioned cultures. Dorn's medium control ■ PBS control ■ Emerged shaft ■ Nonemerged shaft ■ Unsheathed shaft ■.

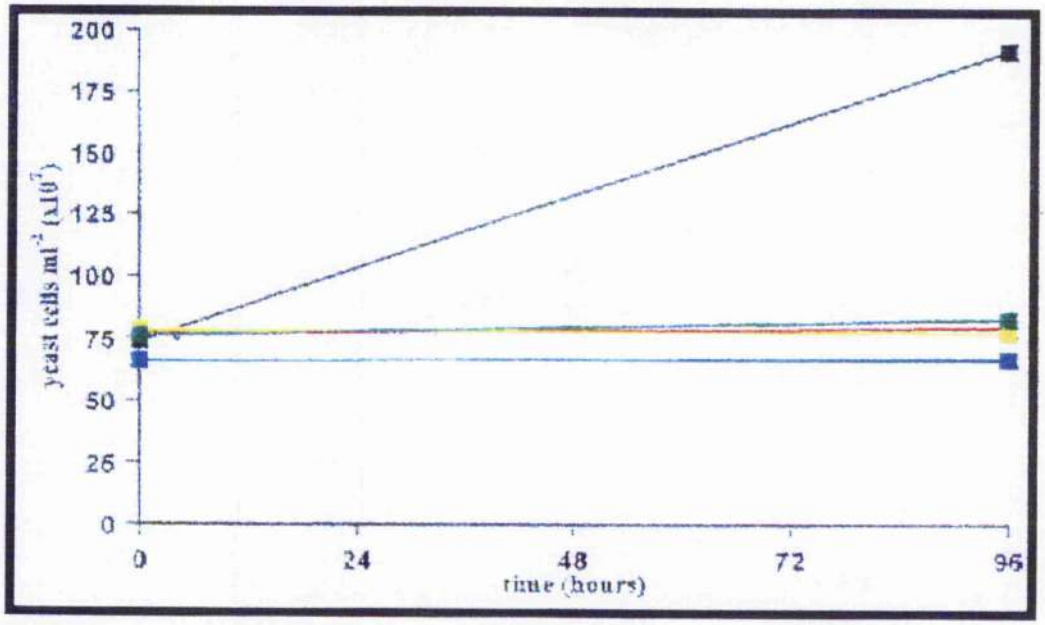

Figure 4.31. Growth of *C. albicans* in follicle component-conditioned PBS. When hair shaft components from donor C were used to condition PBS for 48 hours before the addition of the inoculum, no increase in yeast cell growth was observed over 96h at 29°C . Dorn's medium control ■ PBS control ■ Emerged shaft ■ Nonemerged shaft ■ Unsheathed shaft ■.

Specimens and observations			Hair shaft region		
Morphological observations	**Yeast**	**No. of specimens observed**	**Emerged**	**Nonemerged**	**Unsheathed**
Fibrin-like structures	*M. furfur*	12	10/12	12/12	12/12
Yeast cell clumping	*M. furfur*	12	12/12	12/12	12/12
Bud elongation	*M. furfur*	12	12/12	12/12	12/12
Extracellular coating	*M. furfur*	12	12/12	12/12	12/12
Cuticular destruction	*M. furfur*	12	8/12	4/12	4/12
Filament production	*M. furfur*	4	4/4	4/4	4/4
Cuticular penetration by filaments	*M. furfur*	4	0/4	0/4	0/4
Follicular debris and deposits in association with yeast	*M. furfur*	12	12/12	12/12	12/12

Table 4.1. Summary of morphological observations made on floating filter hair shaft samples by scanning electron microscopy, section 4.5.2.

follicle region	number of smallest squares counted	Average number of yeast cells per smallest square
bulb/ORS	200	0.000
stripped shaft	200	0.26 ± 0.13
nonemerged shaft	200	8.99 ±0.18
emerged shaft	200	2.98 ±0.01

Table 4.2. These figures represent the average number of colonising yeast cells per 200 fields of view counted over different regions of the hair shaft. The results are averaged over three separate sets of experimental results. The nonemerged shaft region was found in each case to show the highest number of colonising *M. furfur* yeast cells after incubation.

Specimens and observations			Hair shaft region		
Morphological observations	**Yeast**	**No. of specimens observed**	**Emerged**	**Nonemerged**	**Unsheathed**
Variable yeast morphology	*M. furfur*	3	3/3	3/3	1/3
Colonisation by *M. furfur*	*M. furfur*	3	3/3	3/3	1/3
Extracellular coating	*M. furfur*	3	3/3	3/3	0/3
Association with follicular debris	*M. furfur*	3	2/3	3/3	0/3
Clonisation of cortical tissue	*M. furfur*	3	0/3	0/3	1/3
Penetration of cuticular layer by *M. furfur* filaments	*M. furfur*	3	0/3	0/3	0/3

Table 4.3. Summary of morphological observations made by scanning electron microscopy of colonisation by *M. furfur* of hair shaft components incubated in *M. furfur*/PBS suspensions, section 4.5.3.

Donor	Time between washes	washed or unwashed	yeast	emerged shaft	nonemerged shaft	unsheathed shaft	positive control	negative control
A	12 hours	washed	*M. furfur*	no increase	no increase	no increase	x 2.5	no increase
B	24 hours	washed	*M. furfur*	no increase	no increase	no increase	x 2.0	no increase
B	24 hours	washed	*M. sympodialis*	no increase	no increase	no increase	x 2.5	no increase
C	48 hours	unwashed 5 days	M. furfur	x 2.25	x 3.33	x 2.00	x 3.80	no increase
C	48 hours	washed	*M. furfur*	no increase	x 2.25	no increase	x 2.00	no increase
C	48 hours	washed	*M. sympodialis*	x.1.16	x 1.85	no increase	x 2.20	no increase
D	72 hours	washed	*M. furfur*	x 1.56	x 1.93	no increase	x 2.00	no increase
E	96 hours	washed	*M. furfur*	x 1.73	x 1.91	x 2.0	x 2.33	no increase
E	96 hours	washed	*M. sympodialis*	x 2.38	x 2.16	no increase	x 3.50	no increase

Table 4.4. Growth of *M. furfur* and *M. sympodialis* in the presence of hair shaft components. Summary of growth measured by increase in number of colony forming units between 0 hours and 72 hours, as in section 4.5.5..

CULTURE CONDITIONS AND YEAST EXAMINED				SIZE OF INCREASE IN VIABLE NUMBER OF YEAST CELLS IN EACH CULTURE.				
Donor	Time between washes	washed or unwashed	yeast	emerged shaft	nonemerged shaft	unsheathed shaft	positive control	negative control
C	48 hours	washed	*M. furfur*	x2.33	x2.0	x1.55	x2.33	no increase
C	48 hours	washed	*C. albicans*	no increase	no increase	no increase	x2.40	no increase
D	72 hours	washed	*M. furfur*	x2.30	x2.3	x 1.43	x2.28	no increase
D	72 hours	washed	*M. sympodialis*	x2.38	x2.38	x 1.96	x2.66	no increase

Table 4.5. Summary of growth of *M. furfur*, *M. sympodialis* and *C. albicans* in hair shaft component-conditioned PBS, section 4.5.6. Growth is indicated by the size of increase in number of yeast cells recorded at 0h and 96h.

DONOR/ ORGANISM	WASHED/ UNWASHED	POSITIVE CTRL	EMERGED	NON EMERGED	UNSHEATHED	NEGATIVE CTRL
A/ *M. furfur*	washed	1.61	0.11	0.08	0.09	0.10
B/ *M. furfur*	washed	1.78	0.45	0.61	0.52	0.42
B/*M.sympodialis*	washed	2.56	0.45	0.79	0.62	0.49
C/ *M. furfur*	washed	2.92	1.13	1.98	1.30	0.76
C/ *M. furfur*	unwashed	2.45	1.90	3.03	2.76	1.23
C/ *M. sympodialis*	washed	2.34	2.16	2.15	0.70	0.67
C/ *M. pachydermatis*	washed	2.30	0.72	0.99	0.80	0.39
C/ *C. albicans*	washed	1.89	0.82	0.85	0.78	0.83
D/ *M. furfur*	washed	2.41	2.31	1.74	0.79	0.71
E/ *M. furfur*	washed	1.64	2.31	2.45	1.63	1.36
E/ *M. sympodialis*	washed	2.10	2.50	2.97	1.09	0.80

Table 4.6. Comparisons of ranges and averages of 0 and 72 hour counts as an estimation of the significance of the increases in viable yeast cell number observed in the hair follicle component conditioned PBS experiments. Direct comparisons may be made between the overall proportions gained from examination of the ranges and means of every dataset included in this study.

DONOR/ ORGANISM	POSITIVE CTRL	EMERGED	NONEMERGED	UNSHEATH-ED	NEGATIVE CTRL
DONOR C/ *M. furfur*	2.42	1.86	1.64	2.00	0.86
DONOR D/ *M. furfur*	2.08	1.05	1.78	0.04	0.91
DONOR D/ *M. sympodialis*	2.93	2.51	2.64	1.98	0.79
DONOR D/ *C. albicans*	2.58	1.07	1.04	1.12	0.90

Table 4.7. Comparisons of ranges and averages of 0hour and 96 hour counts as an estimation of the significance of the increases in viable yeast cell number observed in the hair follicle component-conditioned PBS experiments.

4.6 Discussion

This study has shown that *M. furfur* preferentially adheres to and colonises the nonemerged hair shaft and that soluble components of the nonemerged shaft stimulate yeast cell growth. As *Malassezia* yeasts preferentially colonise sebum-rich regions of the skin [153], it is possible that sebum absorbed onto the hair shaft in the follicular infundibulum contributes to the susceptibility of the nonemerged shaft to colonisation. The sebum at this region has not been treated in any way (see chapter 5) and therefore may also be rich in other lipid- and water- soluble components that may be present in the hair shaft.

4.6.1 Morphological observations as assessed by scanning electron microscopy

Scanning electron microscopic examination of inoculated hair shaft components revealed variable morphology of *M. furfur* on the hair shaft. In some instances, elongating buds were observed as well as the characteristic oval-shaped cells with prominent bud scars. The bud scars on the mother cell are a result of repetitive monopolar or sympodial budding and are typical of all *Malassezia* species. A particular morphology could not be associated with any specific follicular region. Differences in cellular morphology have often been noted in tissue or culture [2,88,89], although in other cases, stable morphological variants have been noted [54,154]. In 1996, Gueho *et al* [97] used morphology as one of the parameters for

reclassifying the genera *Malassezia* into seven distinct species. The variation observed in this SEM study however is confined to the infecting species used, *M. furfur*. Although stable morphological species are claimed in the literature, it is highlighted in the new classification that *M. furfur* may show a high degree of pleomorphism [97] compared to the other species. In essence, the pleomorphism observed in this study is likely to be species-specific and not related to the region of the hair shaft colonised.

The extracellular coating associated with some clumps of yeast cells has been noted in previous experiments [24, 218] though its significance is still unknown. It is possible that it may provide a mechanism of attachment to hairshaft components or stratum corneum. Similar coatings are noted in other fungal genera. In particular *Candida albicans* has often been observed to form an extracellular coating as an adherence mechanism aiding the formation of biofilms. Adherence is thought to help the yeast evade flushing from the epithelial surfaces, and is one of the first steps in the *C. albicans* pathogenic cycle. Douglas [65], investigating the role of mannoproteins in the adhesion of *C. albicans* to host surfaces observed that adhesion was increased if the yeast was grown in media containing 500mM galactose. Galactose was thought to stimulate the formation of a surface fibrillar layer which could be released into culture, from which extracellular polymeric material (EP) could be extracted. This EP was found to consist of 65-82% carbohydrate, with small amounts of protein, phosphorus and glucosamine. The EP had a high mannose content, suggesting that it was mannoprotein in nature [65]. This extracellular coating is thought to aid invasion of the fungi from the host's immune system. Houston and Douglas [112] showed that the production of the fibrillar layer promotes *C. albicans* virulence by increasing

resistance to opsonisation by neutrophils. It is therefore important to characterise the coating associated with *Malassezia* isolates to observe whether it shares any properties with the EP of *C. albicans*. There is evidence that adherence to human stratum corneum cells is not important in the etiology of seborrhoeic dermatitis [23,73] although, further investigation is required to observe whether this coating does play a role in the pathogenesis of seborrhoeic dermatitis and dandruff.

Hill *et al* [108] also observed *P. orbiculare* (Malassezia) yeast cells to be associated with follicular debris and sebum deposits by SEM. They observed that in patients with folliculitis, occlusion of the follicles with cell debris, sebum and squamous cells occurred, *Malassezia* cells being associated with occluded areas of the inflamed follicles. They hypothesised that follicular occlusion may be a prerequisite for yeast overgrowth and folliculitis as it was only associated with inflamed follicles and was not present in biopsy specimens obtained from unaffected controls. It is possible that overgrowth of *Malassezia* in other disease states is also closely related to follicular debris, sebum deposits and occlusion. In this study, the presence of these deposits on the nonemerged shaft below the follicular orifice reinforces the hypothesis of an infundibular *Malassezia* reservoir. Lipid abnormalities are often observed in patients' sebum and it is possible that these lipid abnormalities are due to a build up of follicular bacteria due to occlusion and may contribute to *Malassezia*-related disease states.

Penetration of the cortical layer following damage to the cuticular layer may be significant in *M. furfur* pathogenicity as a method of invasion by the filamentous form

of the organism. There is no evidence in the existing literature of invasive mechanisms in *Malassezia* yeasts.

4.6.2 Stimulation of *M. furfur* and *M. sympodialis* in the presence of dissected hairshaft components

Significant growth of *M. furfur* and *M. sympodialis* in the presence of emerged shaft components appears to be associated with the time interval between hair washes and may be due to sebum becoming trapped at the scalp surface, coating the emerged shaft, as well as other non-sebum soluble materials. Washing of the hair may normally remove the sebum coating, which would explain the poor yeast cell growth in cultures containing components from donors A and B. Frequent washing may even remove sebum lipids from the infundibular portion of the hair shaft. In existing studies of dandruff sufferers there is little evidence that 'greasiness' of hair has been taken into account when assessing the differences between dandruff sufferers and normals. MacKee *et al* [153] carried out a study in which they observed a rather close parallel in the microflora of dry scalps and seborrhoeic dermatitis and a lower incidence of *Pityrosporum* (*Malassezia*) species in oily scalps and faces. However, it was thought that this decreased incidence of *Pityrosporum* (*Malassezia*) species was most likely due to the sampling techniques employed. This study was also more concerned with facial rather than scalp microflora and did not provide a link or disprove the theory of increased *Pityrosporum* (*Malassezia*) colonisation associated with increased scalp

oiliness. In earlier studies however, patients, (predominantly women) gave a clear history of increased greasiness with dandruff severity [3]. Other studies of the correlation of oiliness with increased *Malassezia* colonisation and infection have been limited by small population size, therefore more sophisticated assessments are required.

Stimulation of growth of *M. sympodialis* was observed at the same follicular regions for each donor as for *M. furfur*. In cases of tinea versicolor, an anti-seborrhoeic effect imposed by regular bathing and showering has been shown to have a positive effect on patients' symptoms [202]. The results of the present study are consistent with these findings.

When hair was left for five days without washing, a significant rise in viable yeast cell numbers was observed even in cultures containing portions of the hair shaft normally covered by inner root sheath (IRS) and (ORS). This suggests that some sebum components can penetrate into this region of the hair shaft or that other soluble components which are removed by hair washing are involved.

4.6.3 Hair shaft component-conditioned growth of *Malassezia* yeasts

In the study of hairshaft component-conditioned PBS, viable yeast cell growth was observed for all types of conditioned PBS examined. This suggests that stimulatory factors for *M. furfur* and *M. sympodialis* yeast cell growth have been released into the

PBS. Because the majority of *Malassezia* species will not grow *in vitro* without a lipid supplement, it is possible that follicular or shaft surface lipids and fatty acids have been released into the PBS stimulating yeast cell growth. This phenomenon would satisfy the absolute requirement for long-chain fatty acids for *in vitro* growth of *M. furfur* and *M. sympodialis.*

The work carried out in this study has indicated that in the follicular infundibulum an association of sebum components and other soluble components with hair shaft components may contribute to providing a stable ecological niche for the growth of *M. furfur* and *M. sympodialis*. The follicular infundibulum may be observed a reservoir for *Malassezia* yeasts, which suggests that the nonemerged shaft (NES) of plucked hairs should be assessed for the presence of *Malassezia* yeasts and hence a follicular reservoir. This model may help assess whether or not fungal overgrowth is a major difference between the commensal and pathogenic potential. As yet, no concrete evidence exists to show a correlation between yeast cell numbers and clinical severity at the stratum corneum surface levels. Wikler *et al* [235] could not establish a linear link between yeast cell number and clinical severity, although other studies indicate population densities of yeast cells are up to two log stages higher than on unaffected skin in cases of tinea versicolor [115] as well as significant correlation between severity and fungal population in seborrhoeic dermatitis [106]. At the follicular level there is increased colonisation of *Malassezia* in non-inflamed comedones (68%) [117] in cases of acne vulgaris compared to 13% [134] of normal PSUs. Ingham and Cunningham [115] state that the folliculitis caused by *Malassezia* can only be considered if there is quantitative evidence of yeast cell overgrowth compared to controls. It is possible that in dandruff, aswell as an increased scalp

population of *Malassezia,* there is an increased follicular population aswell. The model used in this study would prove useful in assessing this factor.

4.6.4 Methods of assessing the role of sebum in *Malassezia*-related infections.

In future, it may be important to note whether there is a correlation between sebum levels on scalp and hair shaft and dandruff occurrence over a wider patient group, and whether or not increased production of sebum is a prerequisite for dandruff formation or merely a contributory factor. Various methods could be employed to assess these factors. These include measurement of sebum excretion rate by techniques such as nephelometric estimation of sebum washed from a glass cylinder applied to the skin by suction [36], monomolecular layer techniques [119] or by gravimetric assessment of lipids collected by absorbent papers [215]. This latter method is thought to be the most reliable [36]. Other studies could include the direct study of lipogenesis by the sebaceous gland [49] or study of differences in surface lipid composition [50,123].

4.7 CONCLUSIONS

This study has shown that *M. furfur* shows a particular affinity to the most lipid-rich region of the hair shaft and that soluble components can stimulate yeast cell growth. Although the growth observed in this study does not indicate any specific pathogenic mechanism it supports other evidence [35,115,121] for a major host role in *Malassezia*-related diseases and suggests that investigations into the role of occlusion and sebum excretion rate are required to elucidate more information on the pathogenicity of *Malassezia*-related infections.

Chapter5: SEBUM AND ITS EFFECT ON *MALASSEZIA* GROWTH *IN VITRO.*

5.1 SUMMARY

In this study, growth of *M. furfur* was examined in the presence of sebum extracted from the foreheads of healthy volunteers by the swab or paper method. Concentrated sebum samples from two volunteers were observed to stimulate the growth of *M. furfur in vitro* after three days at 37°C. Sebum suspensions were not observed to stimulate growth at concentrations below 10%.

When concentrated sebum samples from Donors A and B were added to dissected hair shaft components, growth of *M. furfur* was observed in cultures containing sebum or sebum and hair shaft components. Growth was also observed in cultures containing oleic acid and hair shaft components, but the addition of hair shaft components to squalene did not stimulate the growth of *M. furfur*. In comparison, in the stripped stratum corneum model, the additon of concentrated sebum, oleic acid or 8% squalene to D-squame skin biopsies stimulated the growth of *M. furfur*. Hairshaft-conditioned sebum added to stripped stratum corneum samples was also observed to stimulate *M. furfur* growth, to a greater extent than the addition of concentrated sebum alone.

Serial strippings of stratum corneum taken from the underside of the forearm were incubated in the presence of *M. furfur* to observe if there was any difference in colonisation at different depths of the stratum corneum in the presence or absence of a concentrated sebum supplement. Under SEM there was no marked differences in

colonisation of the samples from different levels of the stratum corneum, although the unsupplemented strippings showed evidence of *M. furfur* filament formation which the sebum supplemented samples did not.

From this study it is concluded that concentrated sebum associated with hair shafts and stratum corneum may enhance the growth of *M. furfur*. Stratum corneum and hair shaft factors are most likely to be associated with lipids and fatty acids present and the action of the endogenous bacterial population.

5.2 INTRODUCTION.

It is well documented that *Malassezia* yeasts require a lipid supplement for growth *in vitro*. Lanolin [19] and oleic acid of unknown purity [19] have been used as lipid supplements for *in vitro* growth of *Malassezia* yeasts. A study by Wilde and Stewart [236] showed that *Pityrosporum ovale* will only grow if fatty acids of chain length greater than C10 are added to the culture medium. These results confirmed the work of Shifrine and Marr [205] who concluded that *Pityrosporum* species are incapable of synthesising long-chain fatty acids from C2 units, exhibiting a requirement for preformed fatty acids. This unique requirement for C14-C16 saturated fatty acids of *Malassezia* yeasts is thought to arise due to a blocked capacity to synthesise myristic acid [161, 205]. The fatty acid composition of *M. furfur* yeast cells cultured *in vitro* has been shown to be dependent on the lipid supplements present in the growth medium [161,215]. Nazarro-Porro *et al* [161]suggested from the basis of their study

that fatty acids are not essential as an energy source for *M. furfur* and that they are required for membrane synthesis.

With the increasing evidence that lipolytic activity occurs *in vitro* and *in vivo*, various researchers have suggested that yeasts of *M. furfur* produce a lipase that is liberated extracellularly. Catterall in 1978 [41] suggested that *P. orbiculare* possessed an extracellular lipase crucial for nutrition. Using histochemical techniques and electron microscopy, *in vitro* surface lipase activity was apparent as well as lipase activity in transit through the cell wall, and it was speculated that to utilise skin surface lipids this enzyme may play a role in the pathogenicity of pityriasis versicolor infections. However, the enzyme could not be demonstrated in *M. furfur* cells in the stratum corneum obtained from pityriasis versicolor patients and they concluded that this lipase is not likely to be important *in vivo*, although it may be necessary/critical for *in vitro* fungal growth. This could be due to the abundance of free fatty acids in skin lipids. A similar study by Ran in 1993 [181] used α-naphthyl palmitate as a substrate to measure enzyme activity of *M. furfur*. The enzyme was associated with the insoluble fraction of the organism suggesting that the lipase of the yeast cell of *M. furfur* is localised chiefly within the glucan-rich wall and/or membrane system. In this study, a link was also provided between the yeast-hyphal transition and lipase activity which suggested that *M. furfur* lipase plays an important role not only in cell growth but also with regard to pathogenicity. Work by Ricupito *et al* [187] confirmed the findings of Catterall's study [41]. As in Ran's study, a link between lipase and yeast-hyphal transition was noted suggesting that lipase plays an important role not only in cell growth but with regard to pathogenicity. In other systems such as *C. albicans*, phospholipases may be thought of as a potential virulence determinant and is thought

to play an active role in the invasion of host tissue [187]. Nazzaro-Porro *et al* [161] observed that *in vitro, M. furfur* oxidises unsaturated fatty acids, unsaturated triglycerides, squalene and cholesterol. They hypothesised that *M. furfur* must produce at least one enzyme with lipoxygenase activity. Although increased levels of lipoperoxides and their products were demonstrated from lesional skin of pityriasis versicolor, there was no further evidence that lipoxygenase activity occurred *in vitro*.

Skin surface lipids are derived principally from two different sources. In adults, sebaceous gland activity accounts for the majority of lipids on the skin surface. It has been shown that the amount of surface lipid on a particular area of the skin reflects the size and density distribution of the sebaceous glands in that area [193]. Sebum is produced by a holocrine process in which the sebocytes synthesise lipid as they move towards the centre of the sebaceous gland where they eventually disintegrate, liberating lipids. These lipids can then be discharged via the sebaceous duct into the pilosebaceous follicle from where they are discharged onto the skin surface.

The epidermal contribution of lipids to the skin surface basically consists of triglycerides, cholesterol and cholesterol esters compared to sebum which normally consists of triglycerides, wax esters and squalene. Epidermal lipids are liberated in the keratinization process of the stratum corneum as keratinocytes differentiate.In the past, it has proved difficult to make an assessment of the contributing compositions of the skin surface lipids and sebum lipids. Depending upon which method of investigation is used, there is a propensity to isolate either sebum lipids or skin surface lipids. Since the keratinocyte-derived lipid is essentially an intracellular material, the amount obtained in a sample will probably depend upon the method of collection.

Some of these methods are discussed below. Greene *et al* [95]showed that the quantity of extractable epidermal lipid is between 5 and 10mg per sq cm compared with 150 to 300 mg of sebum per sq cm on the forehead. The epidermal lipid therefore contributes about 3 to 6% of the surface lipid on the forehead.

Other lipids that are present on the skin surface may be looked at as contaminating lipids coming from environmental sources such as soaps, cosmetics, topical medicaments and saturated atmospheric hydrocarbons from petroleum [66,67]. Resident bacterial populations also produce fatty acids and lipids by the action of specific enzymes called lipases. Taking all contributing factors into consideration, we can see that the skin surface lipidic environment creates a dynamic microecology which will vary between the donors examined

Various methods of studying sebum excretion rate and surface lipid composition exist. Sebum excretion rate may be assessed using various techniques such as nephelometric estimation of sebum washed from a glass cylinder applied to the skin surface by suction [36], and monomolecular layer techniques which are based upon the assumption that water insoluble fatty acids as an aqueous surface form a film which is one molecule in thickness. Displacement of a water surface film of mineral oil by a drop of skin lipid solution can be measured and is thought to be directly proportional to the fatty acid concentration of the sample. Gravimetric analyses, however, appear to be the most reliable method. Skin surface lipids can be removed onto a preweighed vessel, either by swabbing with solvent or by collection on preweighed papers. After a timed collection period, the lipid is extracted in a preweighed container after which time the vessel may be reweighed and the

concentration of sebum calculated. Similar methods will be used in this study [104, 119, 215].

Since the surface lipid is a mixture derived from sebaceous glands and keratinocytes, its composition varies with the anatomical site of origin, with areas with a low density of sebaceous glands having a higher cholesterol content and a lower squalene content than the sebaceous-rich areas [95]. To assess lipid composition variation, a variety of methods may be employed and the lipids collected may vary depending upon which method of collection is used. Three commonly used methods of sampling surface lipid have been the use of absorbent papers [50], wiping with a polyurethane sponge soaked in solvent [180] and the direct application of solvent in a glass cylinder pressed firmly to the skin surface [95]. On comparing the three techniques it was found that the samples obtained by the sponge method had a greater free fatty acid content than those obtained by the other methods, possibly due to increased bacterial contamination. The paper collection method appears to yield samples with a lower epidermal contribution, and it has the advantage that the sample can be weighed prior to investigation. The cylinder method using hexane or ether as a solvent tends to isolate more fatty acids than the two other methods and may be preferable to use because of this fact. Factors which each method have in common however include the preparation of the skin surface prior to sampling. It is better to take samples at constant time intervals during the day the same time after preparation prior to sampling. Standardising washing procedures, environmental conditions and sampling methods for each experiment is necessary to allow for statistically accurate comparisons.

Various studies have assessed the differences in chemical composition of human skin surface lipids. Downing *et al* [66] analysed skin surface lipid composition by quantitative thin-layer chromatography. Large differences were observed between individuals in the degree of hydrolysis of triglycerides to free fatty acids. This study supported the earlier observations by Haahti *et al* [100]. It was also observed that the concentrations and composition of cholesterol, wax esters and squalene did not vary appreciably between the different individuals assessed.

Stoughton [214] suggested that

> " The first line of defence of the skin is a thin film of emulsified material spread evenly over its entire surface. This even, pliable film contributes to many essential functions among which are: antisepsis, interference with absorption of toxic agents, buffering of acid and alkali, lubrication of horny layer and control of hydration" [214].

Various functions have been bestowed upon the formation of skin surface lipids. Apart from the list of functions summarised in the "The human integument" [214] other functions bestowed upon sebum lipids (which make up a major part of the skin lipid composition) include the presence of Vitamin D precursor. A review by Kligman [126] challenged all of these views. He showed that the absence of sebum imposes no deleterious effects on the cosmetic quality of skin and that the ultrafilm of sebum found on the skin surface is not thick enough to provide barrier protection for the skin against water loss [27,34]. Post pubertal sebum has also had antifungal properties bestowed upon it. Rothman *et al* [193] reported sebum as being the cause of greatly increased antifungal power which resided in the free fatty acid fraction and the

infrequency of cases of *M. audouini* tinea capitis in adults. Kligman *et al* [126] showed that the antifungal action of sebum was essentially an *in vitro* phenomenon, which was inhibited when in contact with horny substances and in fact sebum actually plays a greater role in fungal skin surface growth than inhibition.

Kligman [126] concluded that the sebaceous gland is probably an obsolescent appendage due to the vestigial properties of the human hair. From this information then it therefore seems more accurate to focus any studies of sebum on the effect of micro-organism growth and pathogenicity, particularly when the negative deleterious effects of sebum are considered, in particular in acne patients.

Free fatty acids in surface lipids are derived from the lipolysis of triglycerides by esterases of bacterial origin. *In vitro*, it is observed that all major skin surface residents including *Corynebacterium acnes, Pityrosporum* and aerobic cocci all produce lipid-splitting enzymes [41]. A study by Marples *et al* [148] assessed the influence of each skin surface species on the generation of free fatty acids (FFA) in human surface lipids. It was observed that removal of coagulase negative cocci did not lower FFA concentration [148]. When *C. acnes* growth was supressed, the FFA concentration fell sharply when there was a sharp decrease in anaerobic population. The percentage of FFA was correlated strictly with *C. acnes* density [148]. A role for yeasts in the generation of free fatty acids was implicated at low *C. acnes* populations. The increased population of *C. acnes* was also observed to increase as *Pityrosporum* populations decreased.

5.3 AIM OF STUDY

In this study, the role of sebum in growth of *M. furfur* has been studied. Between-donor differences in growth stimulation have also been assessed. Extracted sebum in the presence or absence of hair shaft samples or stripped stratum corneum samples has been investigated for potential roles in growth stimulation of *M. furfur*. The aim of this study is to further elucidate factors affecting *M. furfur* growth and proliferation *in vivo* and *in vitro*.

5.4 METHODS

5.4.1 Organisms and culture

Overnight cultures of *M. furfur* (Hook) in Dixon's broth were grown at 29°C. Yeast cells were then washed in PBS containing 0.1% alcohol three times in a bench centrifuge at x1500g. The inoculum was then resuspended in PBS alone and was agitated with glass beads until a single cell suspension was achieved. By counting with a haemocytometer, the number of yeast cells ml^{-1} could be calculated. The suspension was then diluted to approximately $5x10^3$ cell ml^{-1}.

5.4.2 Lipid Preparations

An 8% squalene solution in PBS was prepared and sterilised at 10lbs for 10 minutes. A 10% Tween 80 solution in PBS was prepared and sterilised at 15lbs for 15 min. A 10% oleic acid solution of in PBS was prepared and sterilised at 10lbs for 10min. 10x Phosphate buffered saline (PBS), supplied by Dulbecco was achieved by dilution and filter sterilised.

5.4.3 Collection of sebum lipids from forehead by swabbing.

Cotton wool swabs were pre-treated with hexane and dried before use. The swabs were then dipped in hexane and the forehead of each donor was swabbed in a vertical manner ten times and subsequently extracted.

5.4.4 Paper extraction of sebum from volunteers foreheads.

'Rizla' cigarette papers were initially soaked for 60 minutes in hexane to defat the papers. Once the papers had air dried, piles of ten papers were secured to the forehead with a rubber bandage and were left in place for three hours. The sebum-containing paper samples were then either used directly as a growth substrate or extracted in two

volumes of five millilitres of hexane, with a nitrogen evaporation between each extraction. The extracted sebum was then treated in the same manner as in section 5.4.5.

5.4.5 Sebum Extraction

Swabs and paper-collected sebum samples were extracted in two volumes of five millilitres of Analar hexane (Sigma) for two hours in a clean, sterile preweighed glass vessel. The residual hexane was then evaporated at 29°C using nitrogen gas. Three millilitres of hexane were then used to wash any residual sebum from the vessel walls which was then evaporated to concentrate the sebum at the bottom of the vessel. The tubes could then be reweighed and the amount of sebum was calculated gravimetrically. Once the weight had been established, the lipids were resuspended in 3ml PBS by sonication for five minutes. Aliquots of 180µl of the sebum suspensions were placed in eppendorf tubes and 20µl suspensions *of M. furfur* in PBS were added to achieve a final concentration of $1x10^7$yeast cells $ml^{-1}$and cultures were then incubated at 29°C for 72h. At 0h , 72h and 96h, growth of *M. furfur* was measured by viability counts from 100µl aliquots of serial dilutions plated out and incubated on Dixon's agar at 37°C for three days. Dorn's medium was used as a positive control and distilled H_2O was used as a negative control. Donors A-F were normal healthy individuals who all washed their hair approximately three times weekly. No donor had a history of *Malassezia*-related infection.

5.4.6 Sebum concentration

after the sebumwas resuspended in PBS by sonication, the samples could be split into 1ml aliquots and centrifuged at 11,000 for five minutes. This separated the lipid suspension into biphasic layers, with the sebum components being found in the uppermost layer.

Daily swab samples were taken from donors and stored overnight at -20°C in hexane. After five days, the samples were pooled and could then be extracted as in section 5.4.5. Once the lipids were resuspended in PBS, the solution was centrifuged at x13,000g for five minutes to separate the solution into an aqueous (lower) and non-aqueous (upper) phase. The collected lipids could then be removed from the top phase by pipetting, providing a concentrated sample. To examine the effect of concentrated sebum alone on the growth of *Malassezia*, 180µl aliquots of concentrated sebum were placed in eppindorphs and 20µl suspensions *of M. furfur* in PBS were added to achieve a final concentration of $1x10^7$yeast cells $ml^{-1}$and cultures were then incubated at 29°C for 72h. At 0h and 72h, growth of *M. furfur* was measured by viability counts from 100µl aliquots of serial dilutions plated out and incubated on Dixon's agar at 37°C for three days. The stocks of concentrated sebum were also diluted by 10^{-1} and 10^{-2} and treated in the same manner as the concentrated samples.

5.4.7 Conditioned sebum

Once sebum was extracted and resuspended, 20 intact dissected follicles ,which were assumed to have minimal endogenous yeast colonisation due to rigourous washing and examination, were added to the sebum and were incubated for 72h at 29°C after which time the hair shaft components were removed and the sebum was deemed "conditioned". The conditioned sebum was then divided into 180µl aliquots to which 20µl PBS inocula containing *M. furfur* could be added. Samples were then incubated at 29°C for three days with viable growth being measured by counting viable yeast cell numbers (colony forming units) being measured at 0h and 72h as explained in section 5.4.6.

5.4.8 Hair-follicle conditioned sebum and stripped stratum corneum (ssc) samples.

Fifty microlitres of conditioned sebum was added to SSC D-squame biopsies. The sebum (or lipid) was left for 30 minutes to allow penetration of the stratum corneum. Samples could then be inoculated with *M. furfur* yeast cells and incubated at 37°C for three days in a humidified chamber as in section 2.4.3. At 0h and 72h, SSC samples were placed in five ml aliquots of PBS colonising yeast cells removed by agitation and sonication. These suspension were then serially diluted in PBS, and viable growth counts were carried out by the method employed in section 5.4.6.

5.4.9 Concentrated sebum added to dissected shaft components.

Anagen hair follicles were plucked from the newly washed hair and microdissected to obtain 20-40 intact follicles. 180µl lipid or sebum solutions were then added to eppindorphs each containing five dissected follicle components. A 20µl inoculum containing $1x10^5$ yeast cells ml^{-1} in PBS was then added, and the cultures were incubated at 29°C for three days, with viability being measured at 0h and 72h. Diluted aliquots of each culture were plated onto Dixon's agar and incubated for three days at 37°C and then the number of viable colonies was counted.

5.4.10 Serial strippings

D-squames were depressed five times against the underside of the forearm until no longer sticky. Each D-squame was positioned precisely on the forearm to achieve serial strippings at the same site. Serial strippings with twelve D-squames revealed the living cell layers. D-squames were then cut in half. One half of each D-squame was then supplemented with concentrated sebum whilst the other was not. *M. furfur* suspensions (20µl) were then inoculated onto all the D-squame surfaces and the

samples were then incubated in a humidified chamber at 37°C for 4 days after which time they were fixed and processed for SEM. In preliminary examination of uninoculated serial strippings, no endogenous yeast cells were revealed.

5.4.11 Statistical analyses

As in chapter 4, conventional methods of statistical analyses could not be used due to the limitations imposed by the size of population used and the many sources of variation. The data generated in section 5.5 was interpreted by expressing each zero hour average as a proportion of the 72h total average, as explained in section 4.4.10. These results are shown in Table 5.1.

5.5 RESULTS

5.5.1 Growth of *M. furfur* in sebum suspensions collected by swabbing and extracted with hexane.

For six donors examined, there was no increase in growth of *M. furfur* over 96h. Increase in growth was observed in Dorn's medium without glycine which was used as a positive control, whereas decreases were observed in all samples and negative (PBS-only) control. For each donor, the concentration of sebum had been calculated

gravimetrically before resuspension in PBS. Once resuspended, the sebum concentration for each donor was 5.3% for donor A; 3.6% for donor B; 4.8% for donor C; 3.2% for donor D; 2.1% for donor E and 1.6% for donor F (Figure. 5.1, Figure 5.2).

5.5.2 Growth of *M. furfur* in concentrated sebum collected by hexane swabbing.

Sebum was collected and concentrated as in the method outlined in sections 5.4.5.and 5.4.6. For donors A and B, an increase in viable yeast cells growth was observed in the concentrated sebum cultures.When the concentrated sebum was diluted by 10^{-1} and 10^{-2} dilution, increase in yeast cell growth was observed in the 10^{-1} culture for donor A and donor B. Since the concentrated sebum sample was thought to represent an approximate concentration of 100% the 10^{-1} dilution was regarded as approximating a 10% sebum solution. For the 10^{-2}dilution, viable growth was not observed for either donor (Figure 5.3, Figure 5.4).

5.5.3 Sebum and other lipids added to dissected hair shafts

For donors A and B, stimulation of viable yeast cell growth was observed in cultures containing sebum or sebum and dissected hair shaft components . A 6 to 8-fold

increase in yeast cell growth was also observed for donors A and B in cultures containing oleic acid and hair. In all the other cultures examined, viable growth was not stimulated. In cultures containing hairs in the presence of squalene or in cultures containing oleic acid only, there appeared to be a "supression" of growth compared to the PBS and hair control (Figure 5.5, Figure 5.6).

5.5.4 Growth of *M. furfur* on lipid-supplemented and concentrated sebum-supplemented stripped stratum corneum samples.

When growth of *M. furfur* was assessed on forearm stratum corneum biopsies collected by the D-squame method from donor A, the greatest increase in viable *M. furfur* growth was observed in samples supplemented with squalene (8 to 11-fold increase). Increases in growth were also observed on samples containing concentrated sebum and oleic acid solution. A significant increase in growth was also observed in the negative control. (Figure 5.7, Figure 5.8.).

5.5.5 Addition of hairshaft-conditioned sebum to the stripped stratum corneum model.

For donor A, a 1.5 to 2.0-fold increase in growth were observed for the SSC samples supplemented with concentrated and conditioned sebum and a significant increase in growth was observed for the negative, lipid-free control. The conditioned sebum

samples appeared to stimulate yeast cell growth better than the concentrated sebum alone (Figure 5.9, Figure 5.10).

5.5.6 Serial strippings

Serial strippings of the underside of forearm were taken as outlined in section 5.4.10. On examination using SEM, there were no obvious differences in colonisation patterns, or invasion by *M. furfur*, observed between the sebum-supplemented strippings and respective controls. In sebum-supplemented samples a elongated, oval and round yeast cells were observed. In some cases at the surface stripping, yeast cells were observed associated with corneocytes (Figure 5.11). In some instances the yeast cells appeared to be buried in some sort of matrix whereas in others no coating was observed (Figure 5.12). Variable morphologies were noted (Figure 5.13, Figure 5.14). We can also observe the regular arrangement of the surface corneocytes (Figure 5.11). These observations show little differences as we progress downwards towards the dermis. Extracellular coatings were also observed.

On stripping 2-12 (Figure 5.15 to Figure 5.17), in some instances, pits in the corneocyte surfaces were observed where yeast cells had presumably once been (Figure 5.16). Electron dense regions around yeast cells could also be noted (Figure 5.17). Yeast cells in the 3rd and 4th strippings appeared also to be able to bury themselves into the keratinising cell surfaces. On the 7th stripping there was evidence that the yeast-hyphal transition had occurred albeit to a very low extent (Figure 5.14).

As the strippings led further into the dermis there appeared to be a higher propensity for the yeast cells to be buried within an extracellular matrix, as well as more clumping of yeast cells (Figure 5.15 to Figure 5.16). The control (non-supplemented samples) showed few differences to the supplemented strippings. Again variable morphology was observed at all of the stratum corneum levels and lipidic coatings over the yeast cell surfaces were apparent (Figure 5.19 to Figure 5.20). The main differences between the samples and controls was that we observed filamentation to a low frequency in all samples from the 2nd stripping downwards (Figure 5.21 to Figure 5.26). There was evidence of filaments burying into the surface of corneocytes (Figure 5.21 to Figure 5.23). Filaments were also observed associated with corneocyte surfaces (Figure 5.24 to Figure 5.26). The filaments observed were irregular in shape and grew from various surface points on the mother cell. Yeast cells were also observed to bury into the corneocyte surfaces (Figure 5.23) as well as being associated with extracellular matrices. Yeast cells remained viable as budding was observed in all samples.

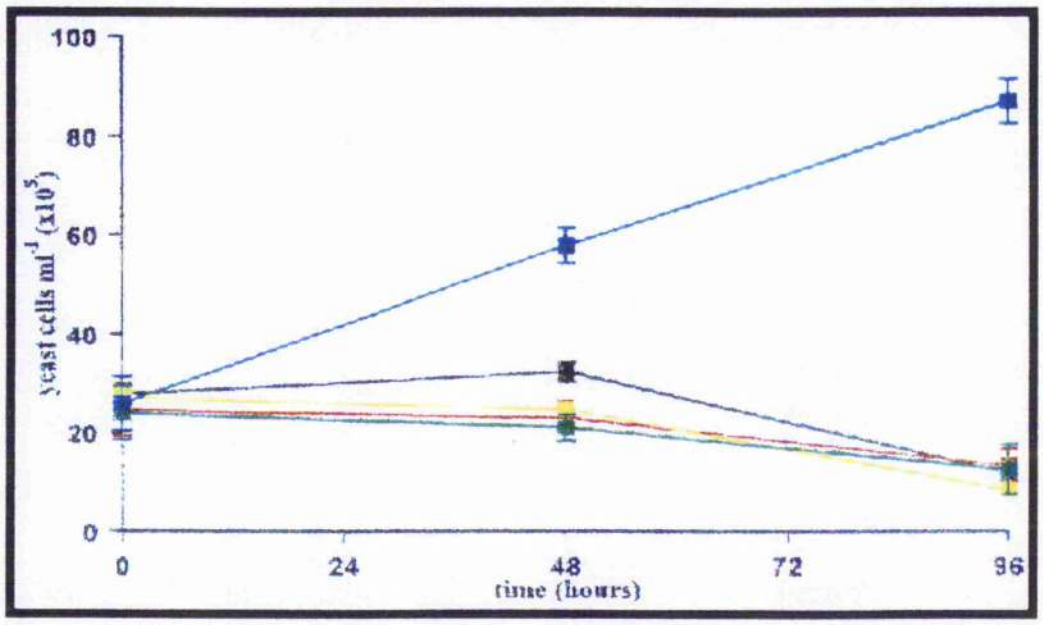

Figure 5.1. Growth of *M. furfur* in sebum solutions collected by swabbing and extracted with hexane. For Donors A, B and C, when the collected sebum samples were resuspended in PBS and incubated in the presence of M. furfur, no stimulation of growth was observed. ■ negative (sebum-free) control ■ positive (Dorn's) control ■ Donor A ■ Donor B ■ Donor C.

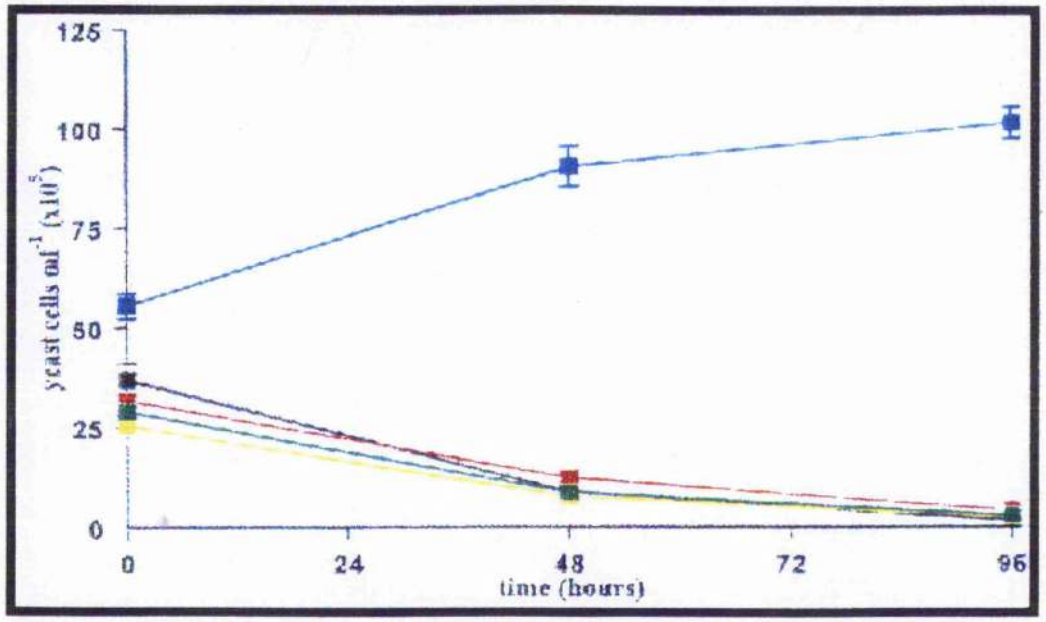

Figure 5.2. Growth of *M. furfur* in sebum solutions collected by swabbing and extracted with hexane. For Donors D, E and F, when the collected sebum samples were resuspended in PBS and incubated in the presence of M. furfur, no stimulation of growth was observed. ■ negative (sebum-free) control ■ positive (Dorn's) control ■ Donor D ■ Donor E ■ Donor F.

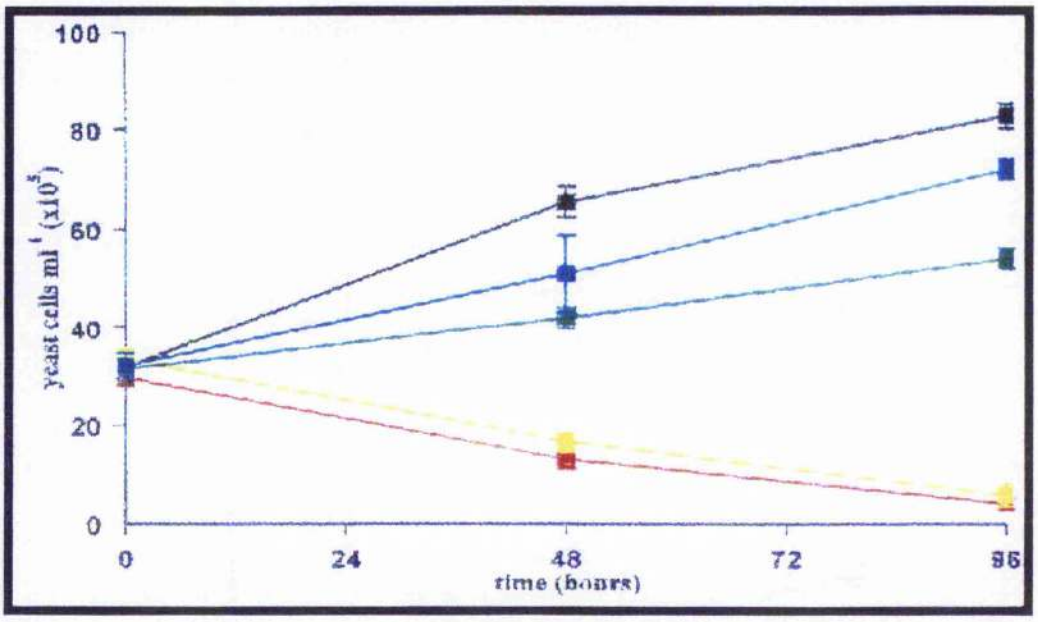

Figure 5.3. Growth of *M. furfur* in concentrated sebum samples collected by swabbing. For Donor A, a significant increase in viable growth was observed in these samples. On dilution, growth was stimulated at approximately 10% but no growth was observed at approximately 1.0% sebum concentration. ■ positive (Dorn's medium) control ■ negative (sebum-free) control ■ concentrated sebum ■ 10% sebum ■ 1.0% sebum.

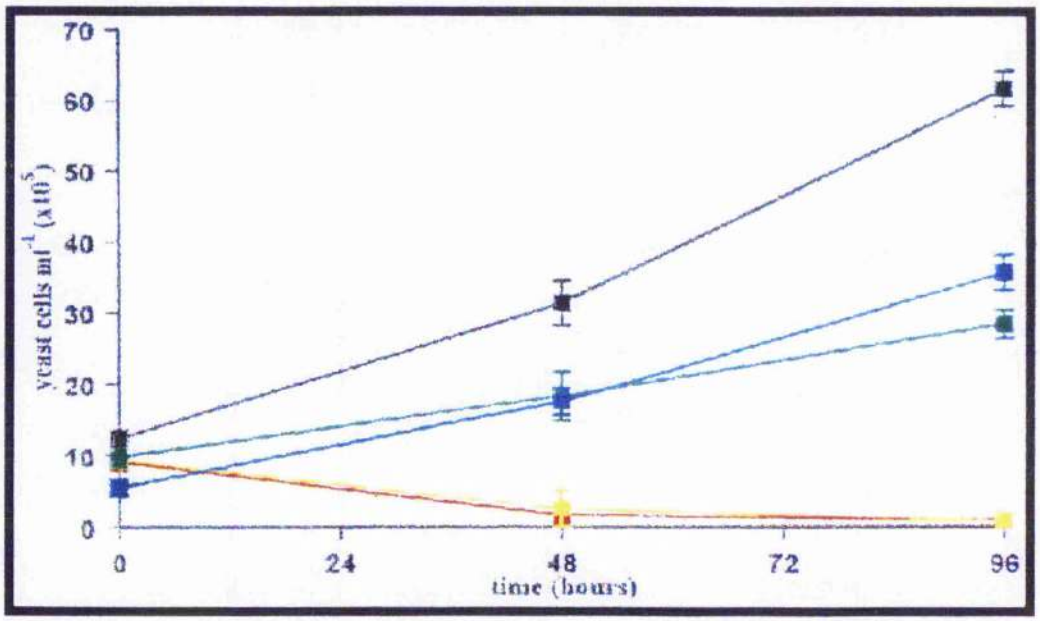

Figure 5.4. Growth of *M. furfur* in concentrated sebum samples collected by swabbing. For Donor B, a significant increase in viable growth was observed in these samples. On dilution, growth was stimulated at approximately 10% but no growth was observed at approximately 1.0% sebum concentration. ■ positive (Dorn's medium) control ■ negative (sebum-free) control ■ concentrated sebum ■ 10% sebum ■ 1.0% sebum.

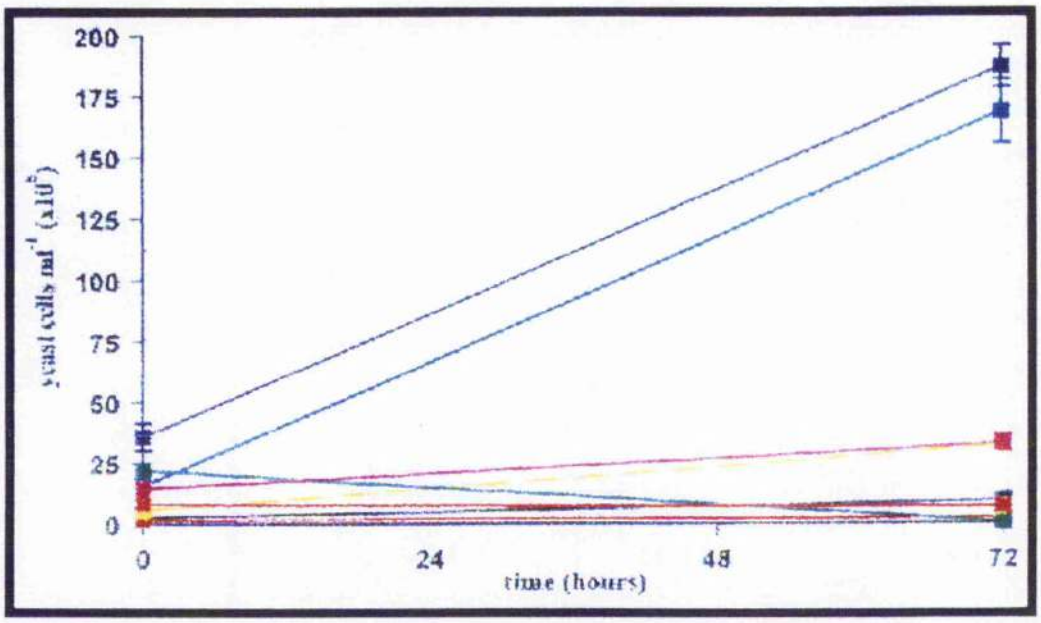

Figure 5.5. Growth of *M. furfur* in sebum- and lipid-supplemented hair shaft samples for donor A. Stimulation of viable yeast cell growth was observed in cultures containing sebum. Growth was also observed in the presence of oleic acid supplemented hair shaft samples. Squalene failed to stimulate viable yeast cell growth under these conditions. Sebum ■ sebum+hair ■ oleic acid ■ oleic acid + hair ■ squalene ■ squalene + hair ■ PBS + hair ■.

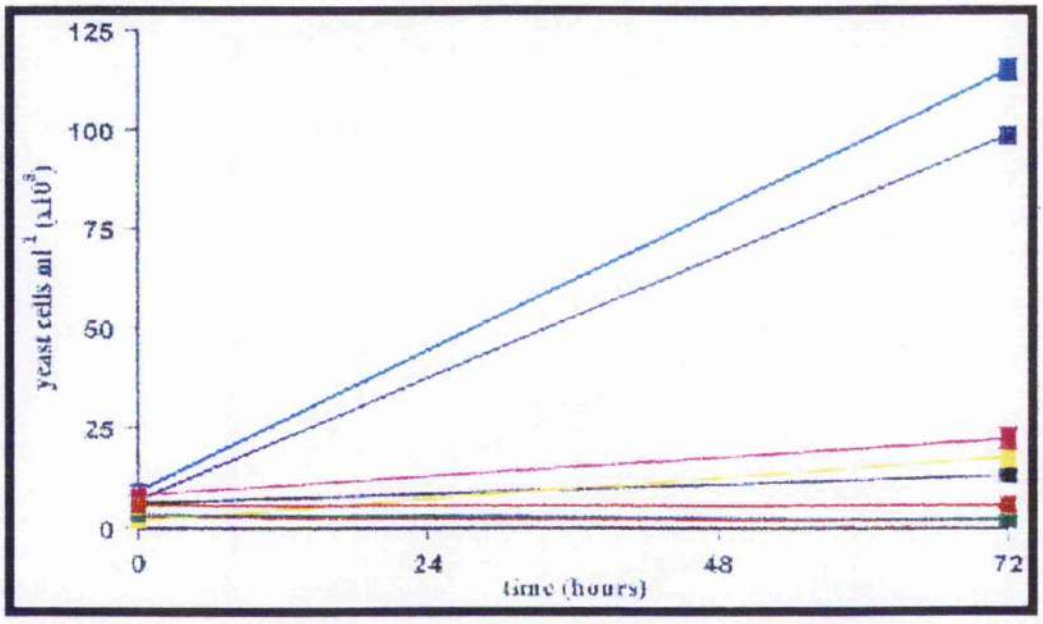

Figure 5.6. Growth of *M. furfur* in sebum- and lipid-supplemented hair shaft samples for donor B. Stimulation of viable yeast cell growth was observed in cultures containing sebum. Growth was also observed in the presence of oleic acid supplemented hair shaft samples. Squalene failed to stimulate viable yeast cell growth under these conditions. Sebum ■ sebum+hair ■ oleic acid ■ oleic acid + hair ■ squalene ■ squalene + hair ■ PBS + hair ■.

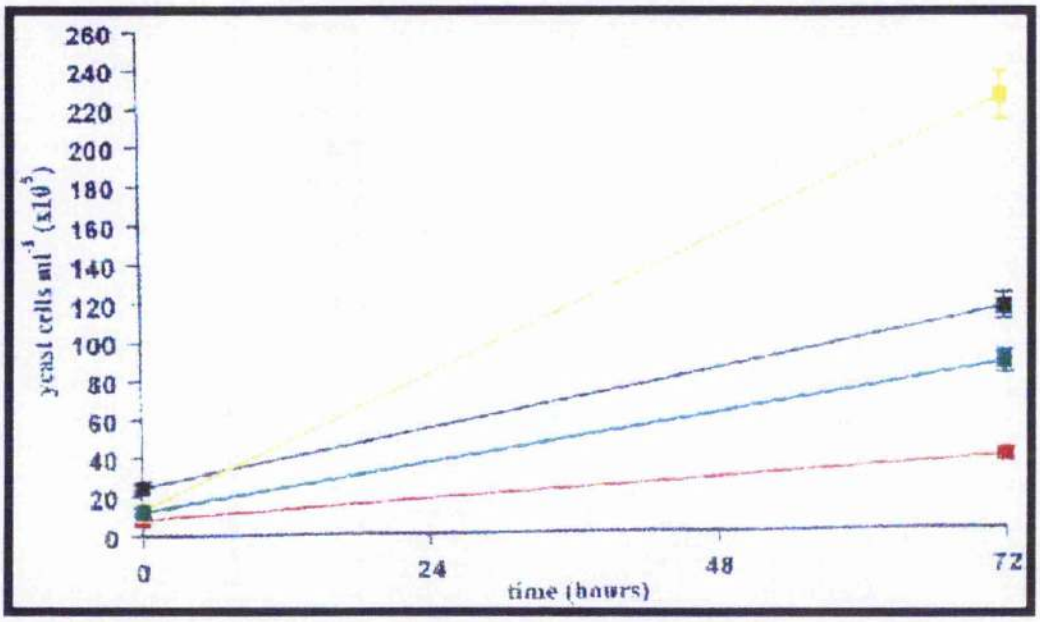

Figure 5.7. Growth of *M. furfur* on lipid-supplemented and concentrated sebum supplemented stripped stratum corneum samples for donor A. Samples supplemented with squalene showed the greatest increases in viable yeast cell number. Highly significant growth was also observed in samples containing concentrated sebum or oleic acid supplements. ■ squalene ■ oleic acid ■ concentrated sebum ■ PBS.

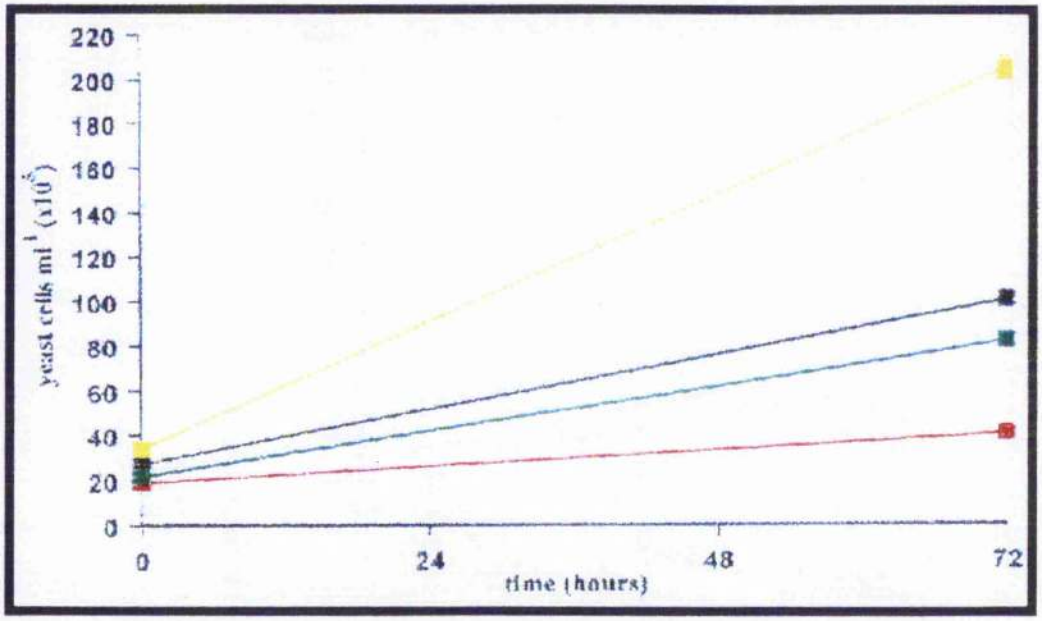

Figure 5.8. Growth of *M. furfur* on lipid-supplemented and concentrated sebum supplemented stripped stratum corneum samples for donor B. Samples supplemented with squalene showed the greatest increases in viable yeast cell number. Highly significant growth was also observed in samples containing concentrated sebum or oleic acid supplements. ■ squalene ■ oleic acid ■ concentrated sebum ■ PBS.

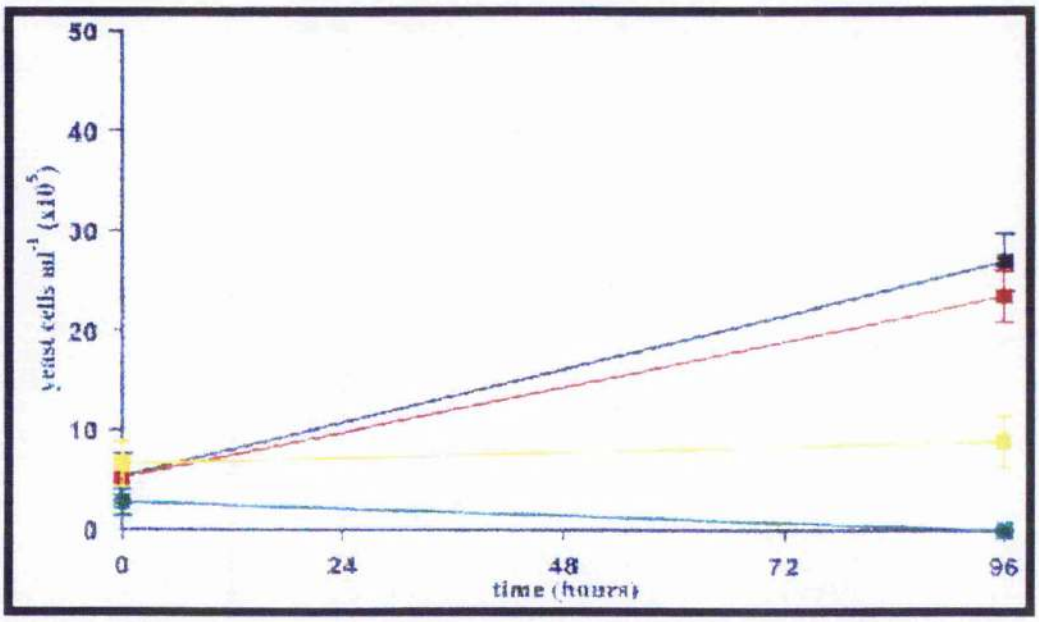

Figure 5.9. Growth of *M. furfur* on stripped stratum corneum sample with concentrated and/or conditioned sebum. Concentrated and conditioned sebum samples were observed to stimulate yeast cell growth in the presence of stripped stratum corneum samples for donor A. ■ conditioned sebum ■ concentrated sebum ■ unsupplemented control ■ D-squame without stratum corneum biopsy.

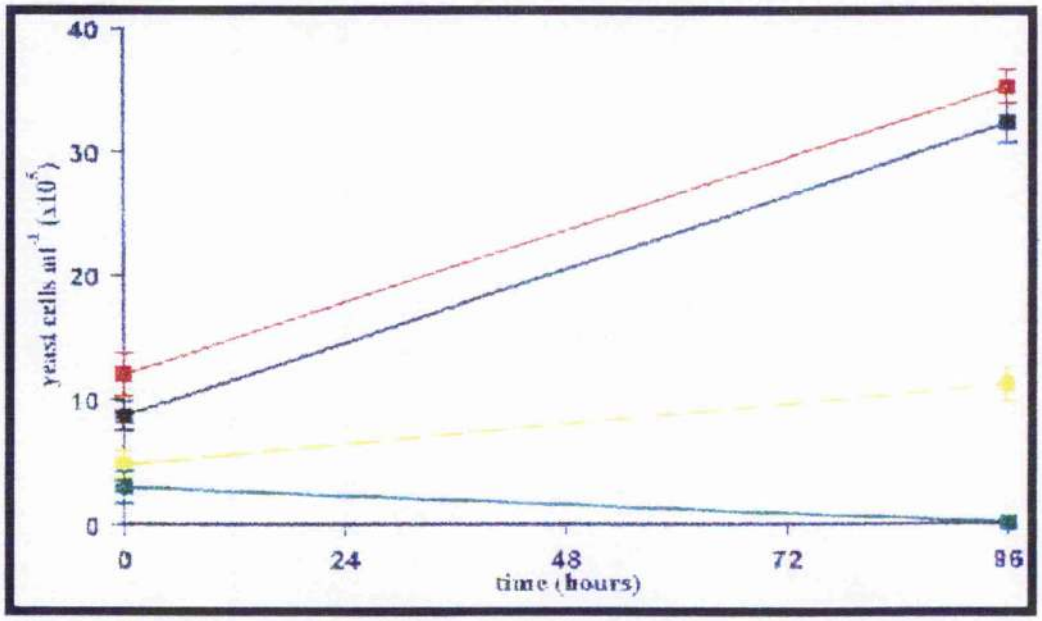

Figure 5.10. Growth of *M. furfur* on stripped stratum corneum sample with concentrated and/or conditioned sebum. Concentrated and conditioned sebum samples were observed to stimulate yeast cell growth in the presence of stripped stratum corneum samples for donor B. ■ conditioned sebum ■ concentrated sebum ■ unsupplemented control ■ D-squame without stratum corneum biopsy.

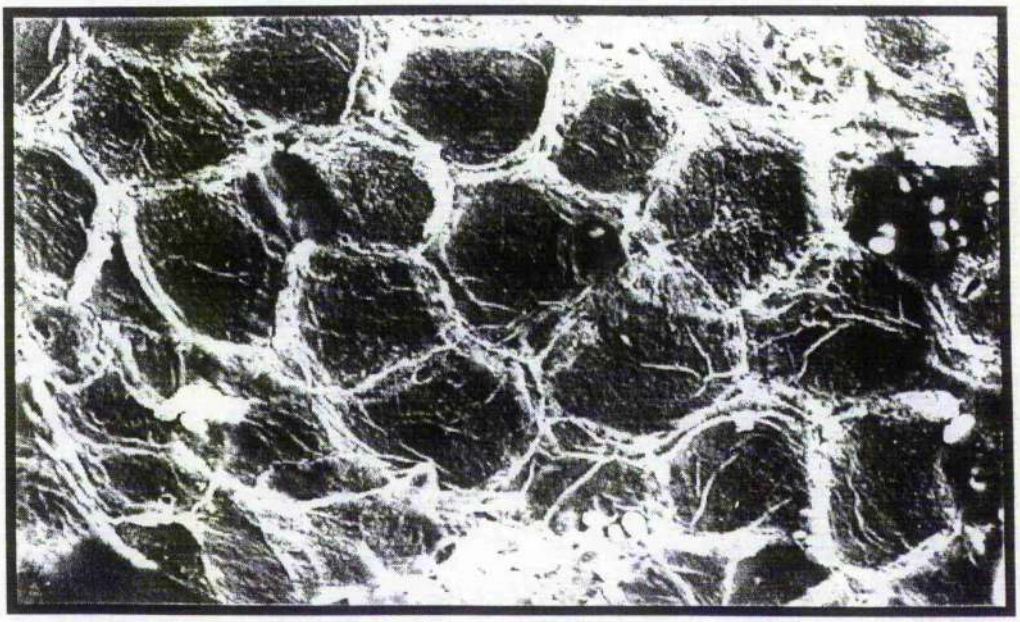

Figure 5.11. *M. furfur* yeast cells associated with corneocytes at the stratum corneum surface (sample 1). Note the regular arrangement of the corneocytes. X1,500.

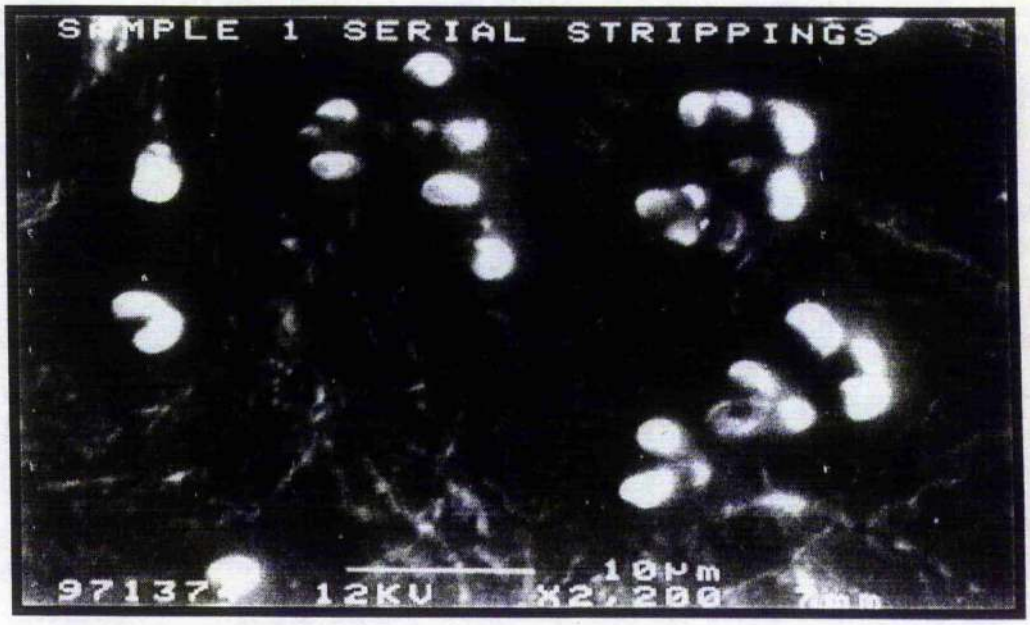

Figure 5.12. Surface stripping from underside of forearm. Yeast cells are associated with an extracellular matrix on the corneocyte surface. X2,200.

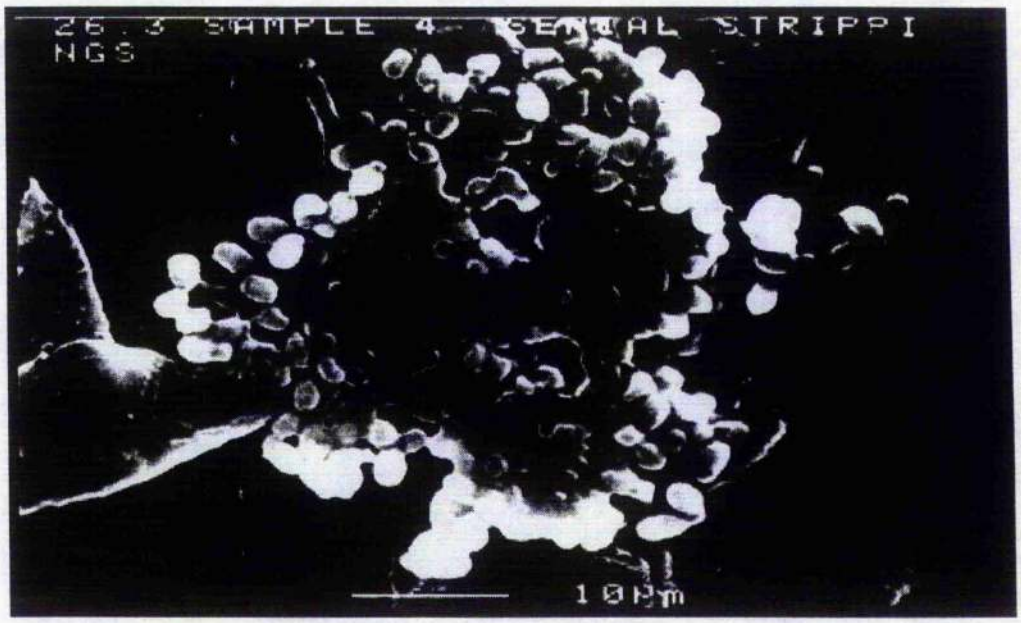

Figure 5.13. Serial strippings of the stratum corneum, stripping 4. Yeast cells clumping appears to increase as strippings are taken from deeper layers of the stratum corneum, with an increased propensity for association with an extracellular matrix.

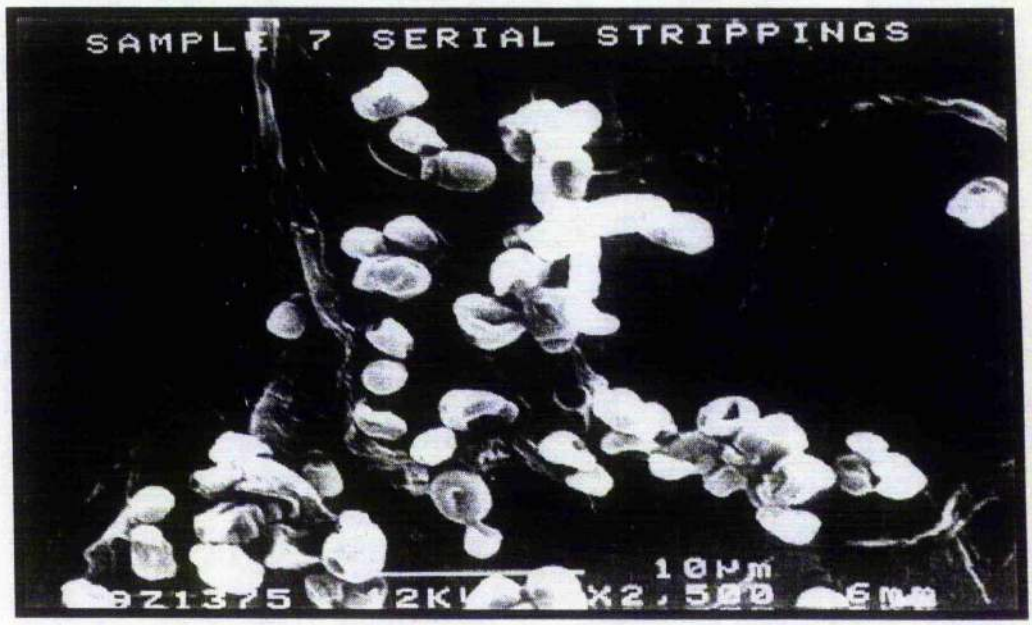

Figure 5.14. Serial strippings of the stratum corneum, stripping 7 (sebum supplemented). Hyphal form was observed in association with clumps of yeast cells. No other evidence of the yeast-hyphal transition was observed. Yeast cells are observed more in clumps rather than as singular cells on the corneocyte surface. X2,500.

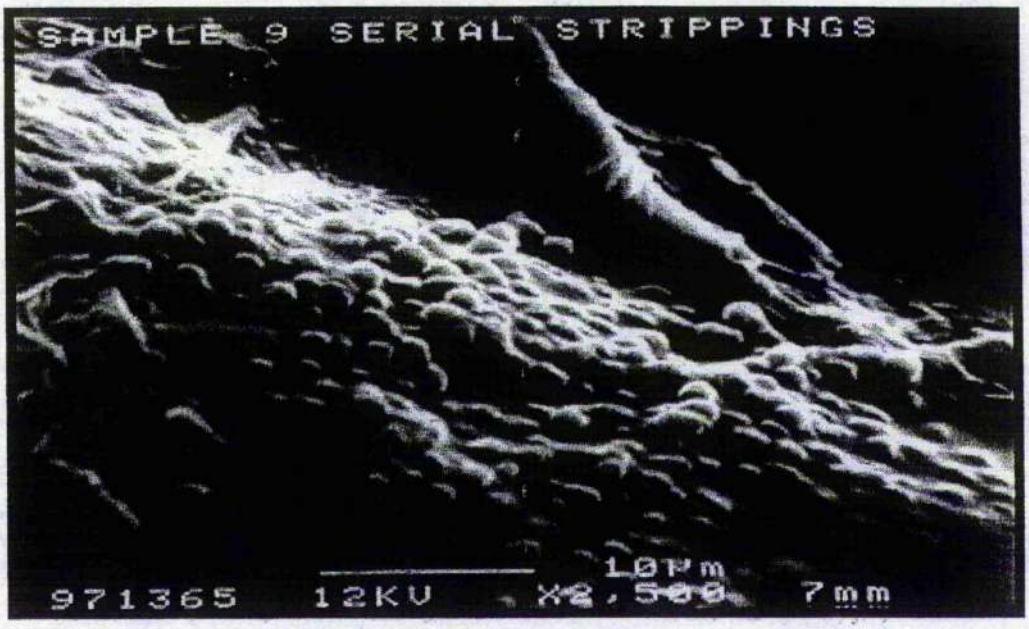

Figure 5.15. Serial stripping number 9, sebum supplemented. Extensive clumping of yeast cells was observed, in comparison to the prior strippings. An extracellular matrix was also observed over the surfaces of all clumps of yeast cells. X2,500.

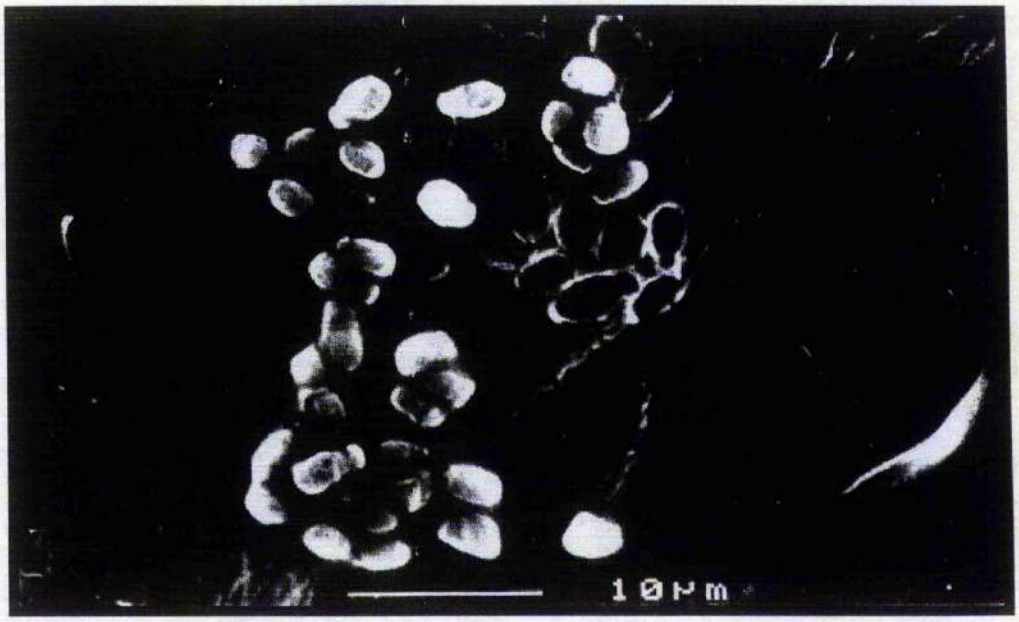

Figure 5.16. Serial stripping number 7, unsupplemented. Yeast cells are observed burying into stratum corneum surface. Pits are observed in corneocyte surfaces where yeast cells have presumably been dislodged. X2,300.

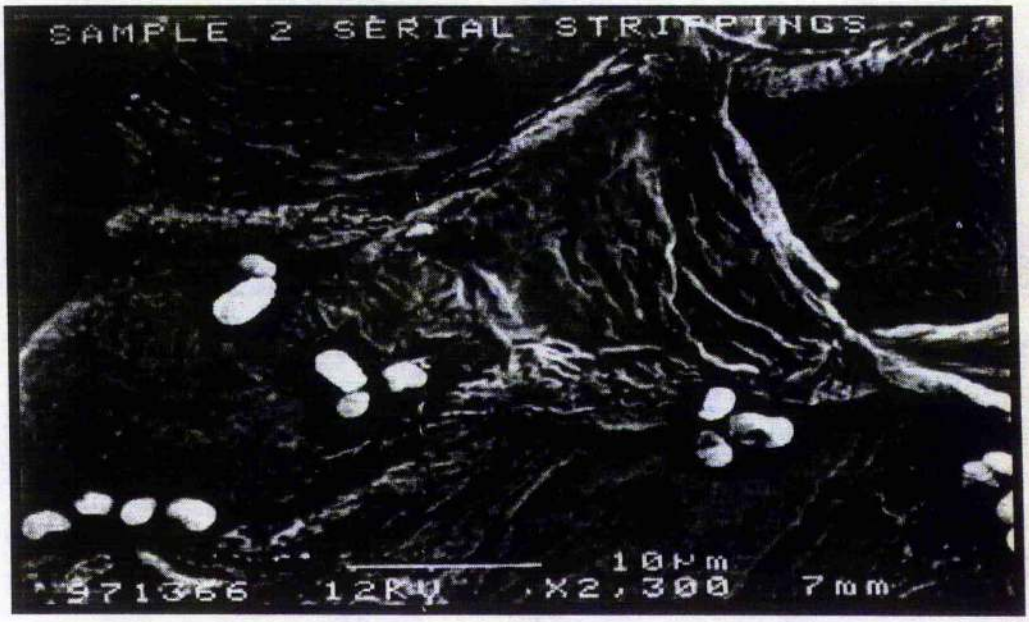

Figure 5.17. Serial stripping 2, unsupplemented. Yeast cells on corneocyte surfaces, surrounded by electron-dense regions. X2,300

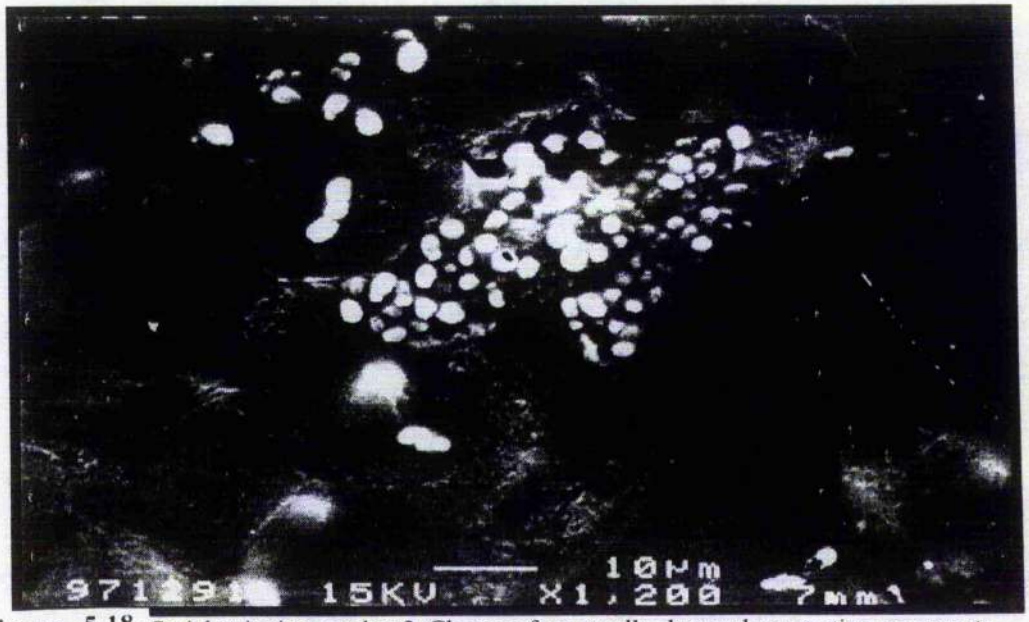

Figure 5.18. Serial stripping number 2. Clumps of yeast cells observed penetrating corneocyte surfaces. X1,200.

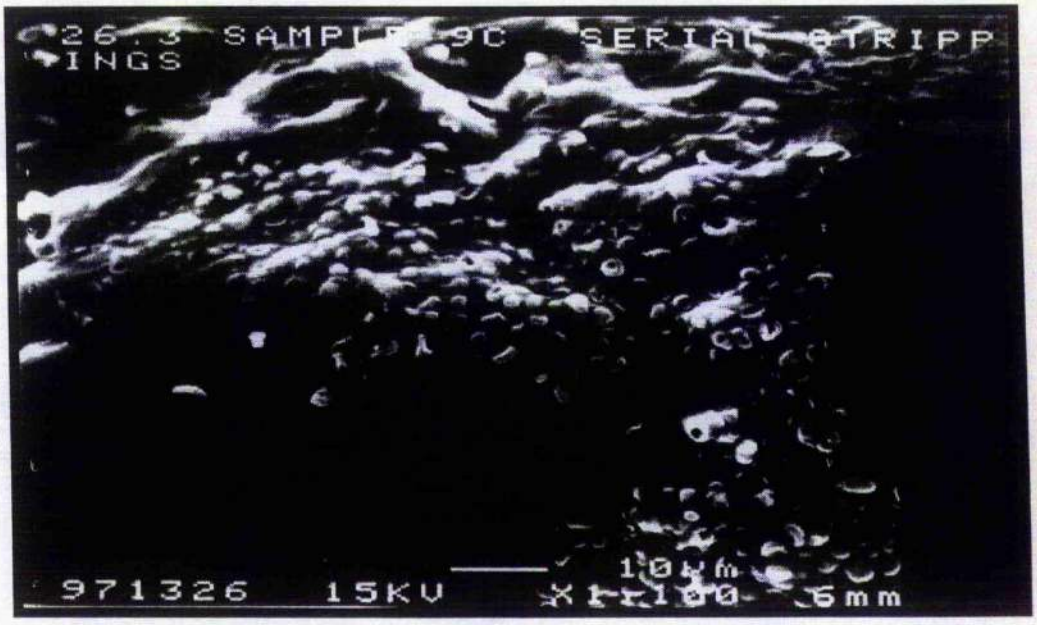

Figure 5.19. Serial stripping number 9, unsupplemented. Extracellular coating associated with clumps of yeast cells. elongated, oval and round yeast cells observed. X1,100.

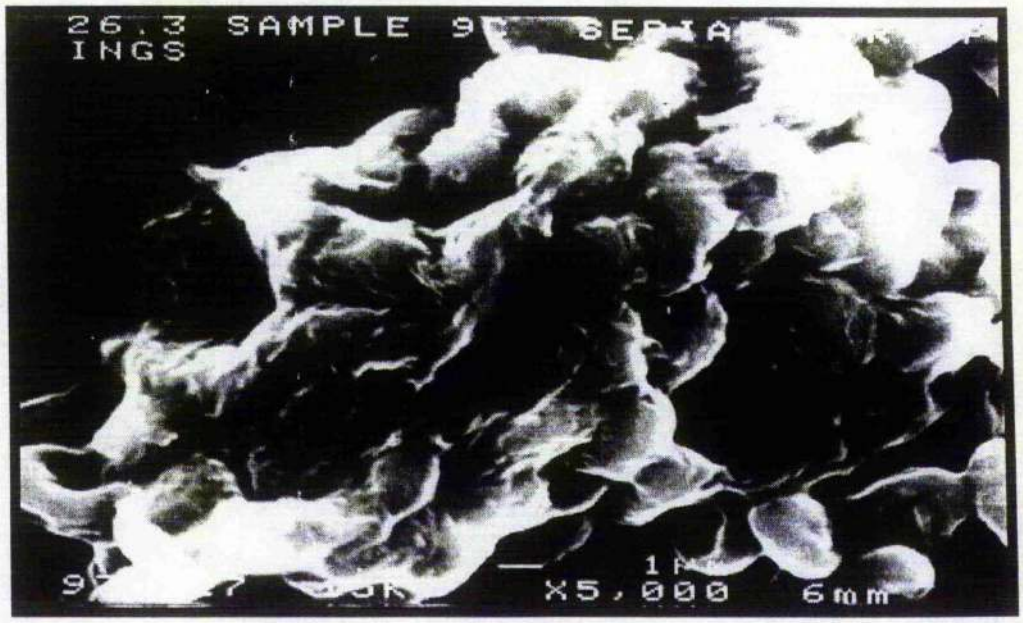

Figure 5.20. Serial stripping, number 9, unsupplemented. Clumps of yeast cells are associated with an extracellular matrix. X5,000.

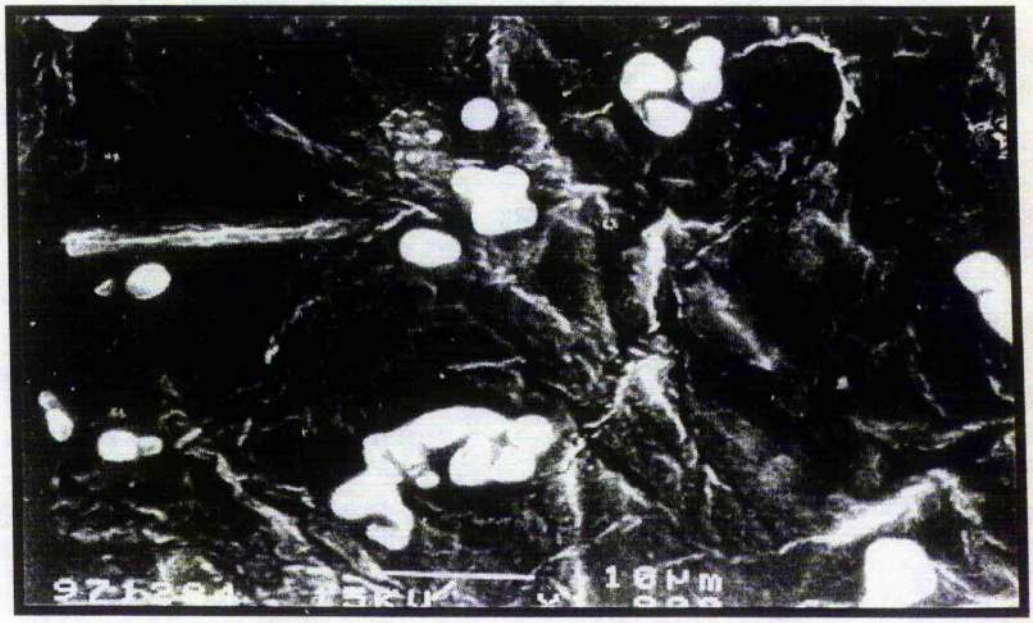

Figure 5.21. Serial stripping number 2, unsupplemented. Clumps of yeast cells associated with the surface of corneocytes. There is evidence of penetration of filaments under corneocyte surfaces. Filamentation observed at a low frequency. X1,900.

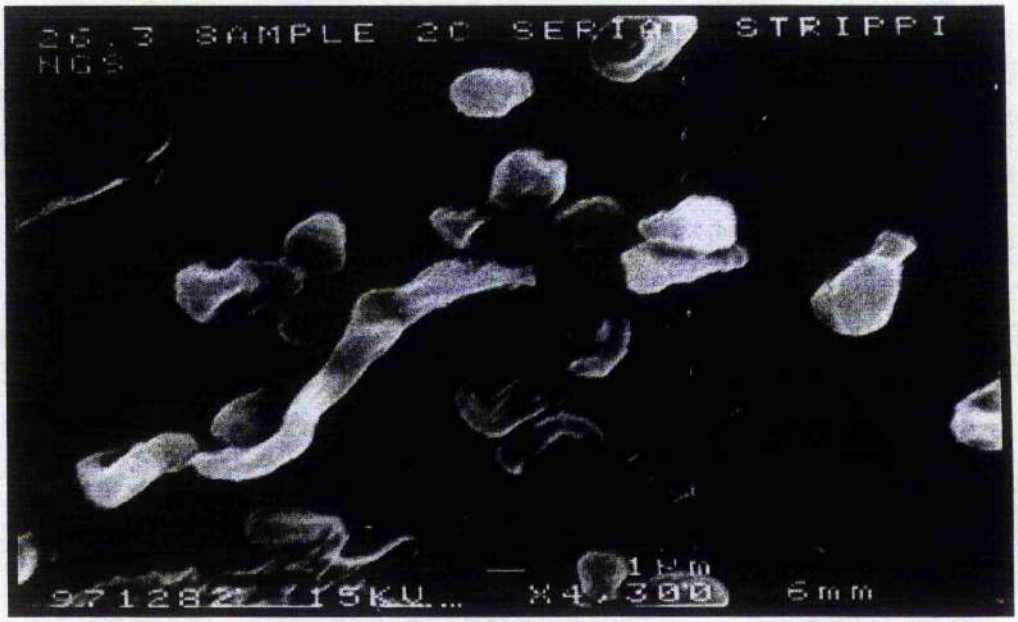

Figure 5.22. Serial stripping, number 2, unsupplemented. Long, irregular shaped filaments are observed in association with yeast cells. X4,300.

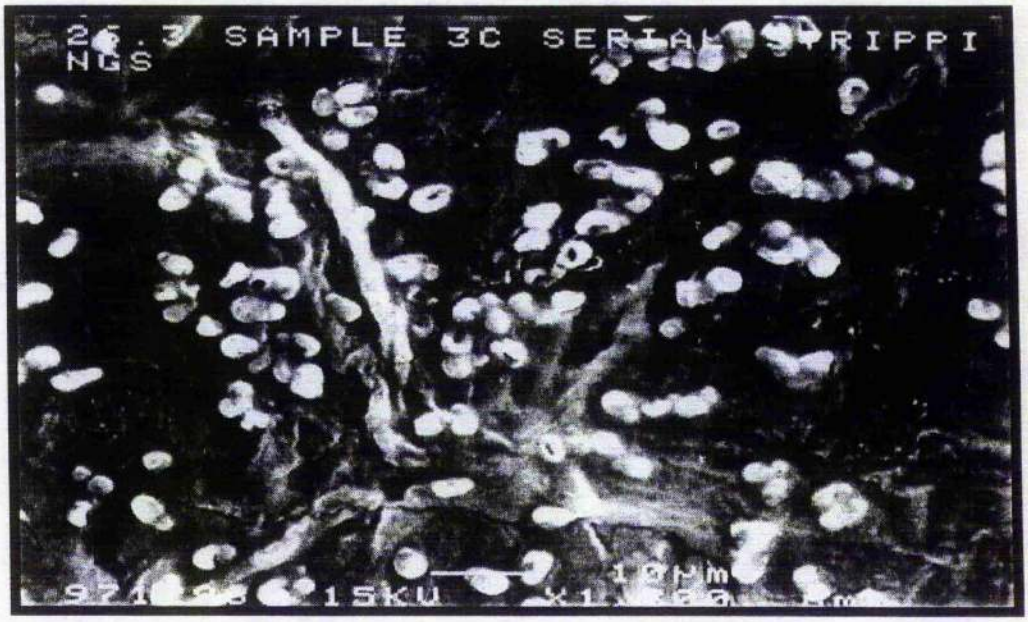

Figure 5.23. Serial strippping, number 3, unsupplemented. Yeast cells and filaments observed, buried into the surface of corneocytes. Yeast cells are mainly observed in clumps. X1,300.

Figure 5.24. Serial stripping number 11, unsupplemented. Filaments observed with clumps of yeast cells. X3,000.

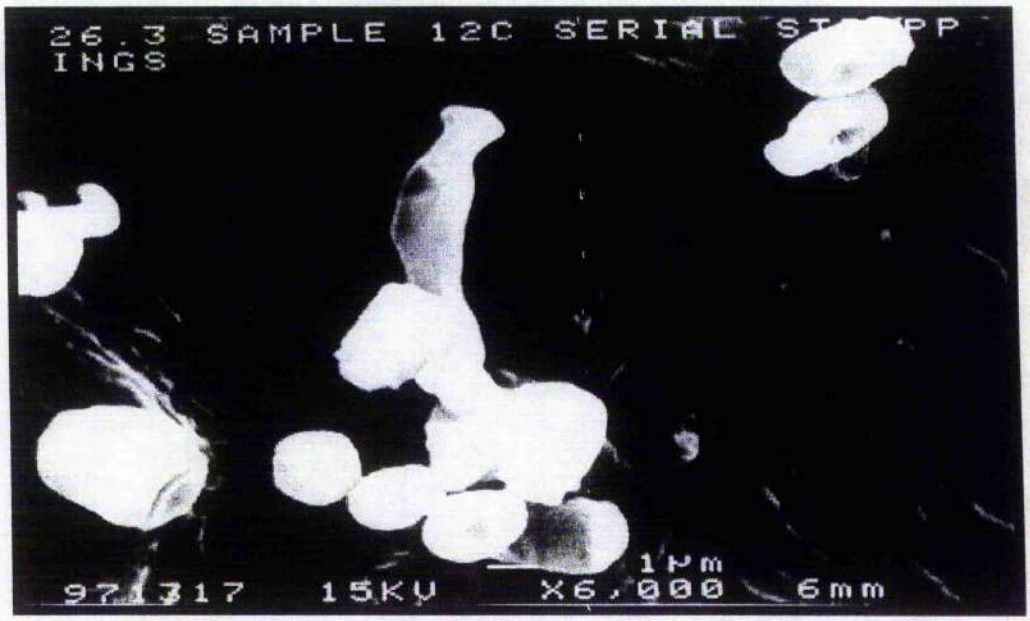

Figure 5.25. Serial stripping number 12, unsupplemented. Filaments observed in association with yeast cells and penetrating corneocyte surfaces. X6,000.

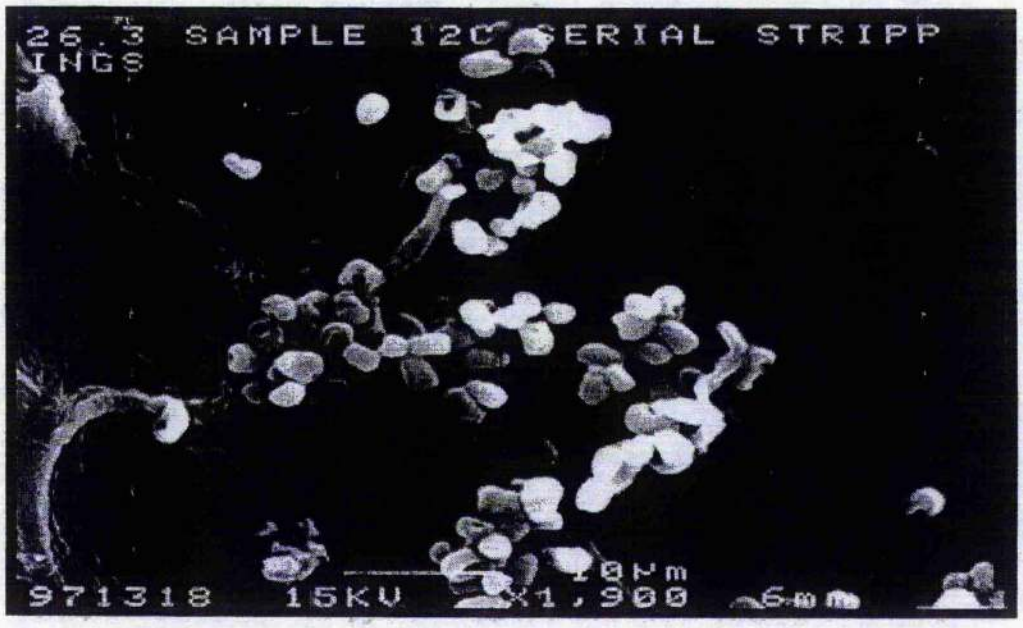

Figure 5.26. Serial stripping number 11, unsupplemented. Clumps of yeast cells observed, burying into corneocyte surfaces. X1,100.

Results expressed as a proportion of the average of 0 hour results		
conditions	**Donor A**	**Donor B**
squalene/hair	0.74	2.45
squalene control	0.19	0.4
oleic acid/hair	2.56	3.32
oleic acid control	0.05	0.43
sebum/hair	13.3	21.44
sebum control	14.74	18.37
PBS/hair	2.6	4.17
PBS control	0.54	1.053
conditioned sebum	5.49	1.7
sebum	4.78	1.67
skin control	1.81	0.46
no skin control	0	0.01
stripped stratum corneum only	2.55	1.58
sebum +stripped stratum corneum	7.77	3.93
squalene + stripped stratum corneum	15.13	7.99
oleic acid + stripped stratum corneum	5.83	3.2
positive control 96h	2.6	4.63
negative control 96h	0.06	0.08
10-2 dilution	0.44	0.05
10-1 dilution	3.2	2.14
concentrated sebum 96h	1.76	2.67

Table 5.1. These are the final comparisons to make to indicate differences between the parameters listed for A and B. These results are based on minimum differences between averages and ranges examined. The results express the differences in increase of cell number between different parameters and donors.

5.6 DISCUSSION

In this study it was observed that sebum at a concentration of no less than 10% stimulated yeast cell growth of *M. furfur* and that the incorporation of sebum into the stripped stratum corneum model or hair shaft model increased the stimulation of *M. furfur* growth.

5.6.1 Growth of *M. furfur* in sebum collected by the swab method.

At low sebum concentrations, *in vitro* growth of *M. furfur* was not stimulated in cultures containing sebum from various donors. When the concentration of sebum was increased above 10%, stimulation of yeast cell growth was observed. For the donors examined, a 2-4 fold increase in yeast cell growth was observed at concentrated sebum values. The increase in growth decreased with decreasing concentrations of sebum. It could therefore be argued that any aqueous liquids on the skin surface may play a role in the control of *M. furfur* growth on the skin surface by diluting the surface concentration of sebum, forming an emulsion where water is dispersed in sebum. The mixture of sweat on the skin surface with sebum may lead to an oil-in-water emulsion or a water-in-oil emulsion, though it has been proposed that this is dependent upon the profuseness of sweating of any individual though it is thought that water-in-sebum is the norm [204]. It was proposed that sebum lipids contained emulsifying agents that worked in 2 different manners. When the aqueous

phase is in excess an oil-in-water emulsion occurs whereas when the lipid phase is in excess a water-in-oil emulsion occurs. The idea of sebum as an emulsifier has been investigated before [173]. Herrman et al [107] showed that spontaneous emulsion of homologous sweat and sebum occurred *in vitro* when drops of sebum were surrounded by sweat. It appeared that a recognition system occurred between sweat and sebum. With other aqueous solutions such as distilled water that were investigated, less emulsification was observed. Hermann *et al* [107] therefore maintained that increasing levels of sweating increased the delivery of sebum across the skin surface. This was thought to aid maintain the plasticity and flexibility of the stratum corneum barrier, which is thought to be dependent on its capacity to hold water [27]. This theory was later disproved by Ikai *et al* [114] showed that the delivery of sebum across the skin surface was more likely to be dependent on temperature and was not influenced by the presence or absence of sweating. It has subsequently been shown that sebum and sweat emulsion do not form naturally on the skin surface. Any emulsion present is transiently formed and disperses almost immediately. It may be that sebum persists in a state described by much earlier investigators [169], as a water-free continuous fatty phase. From this it suggests that by concentrating the sebum in our experiments we have created a more realistic environment with which to examine the effect of sebum upon *M. furfur* growth. Growth of *M. furfur* in cultures containing >10% but less than a 100% sebum concentration may then have been due to a slight partitioning of the solution into the aqueous and nonaqueous phase, concentrating the sebum to stimulate *M. furfur* growth. It may be important to examine the effect of different sweat components on *M. furfur* growth but these are most likely to play minor roles in *M. furfur* growth compared to the role of sebum. Another beneficial aspect of concentrating the sebum

samples before use in this study was that any skin surface debris and endogenous organisms were removed by the centrifugation step which meant that any effects imposed on the growth of *M. furfur* from sources other than the sebum lipids, were minimised.

Differences in sebum composition between the various donors was not examined in this study as all donors observed showed stimulation of growth of *M. furfur* when their sebum was collected by the hexane swab method and concentrated by centrifugation. In this study, hexane was used as the solvent of choice for sampling and extracting sebum lipids. Some schools of thought hold the belief that solvents differ in their extraction properties and that a different array of lipids may be extracted by different solvents [164]. Greene *et al* [95] suggested that the use of water-immiscible solvents for the extractions most likely prevents penetration to the lower epidermal layers and limits extraction to the desiccated surface layers. More polar solvents such as Chloroform:methanol mixture will penetrate to remove lipids from deep in the epidermis [36]. However, Downing and Strauss [67] subsequently showed that the choice of solvents makes a negligible difference to the results. Various methods exist to investigate the composition. As mentioned previously, various sampling techniques exist which show differences in the composition of lipids which they pick up. Burton and Shuster[36] suggests that the differences observed are due to the relative proportions of epidermal and sebaceous lipids obtained by each method. Cunliffe *et al* [51] have investigated this hypothesis by examining differences between the 3 main collection methods [52,66,95, 180]. The sponge method of collection was observed to collect a greater proportion of fatty acids and less triglyceride and an increase in collection of cholesterol by the sponge or cup

technique in comparison to the paper technique. The paper method was observed to contain more wax esters and squalene. These comparisons suggested that the paper method was the most accurate method for sebum sampling as it isolated a lower epidermal proportion of lipids than the other 2 methods. However, Greene [95] stated that the relative contribution of epidermal lipids to the total extractable surface lipids is an insignificant fraction of the total-extractable surface lipid on areas rich in sebaceous glands. Chromatographic methods such as thin layer chromatography or gas-liquid chromatography may be used to separate the different lipid classes. Because we did not observe any vast between-donor differences we did not feel that an analysis of the sebum lipids for each donor would enhance our understanding of our observation at this stage. However, because tried and tested accurate techniques exist, it does open the possibility of looking at differences in lipid composition between a variety of donors if their patterns of *M. furfur* growth stimulation are significantly different. In seborrhoeic dermatitis, both sebum composition and the effect of sebum excretion rate have been studied [22,109] . Only minor abnormalities in concentrations of cholesterol and squalene and unsaturated fatty acids was observed. Therefore it may not be pertinent to study the lipid composition and its effect on *M. furfur* growth any further at this stage between patients and nonpatients.

We did not observe any overproliferation of yeast cell growth in the presence of sebum alone, although this may be expected as the sebum used at concentrated levels should not differ greatly from sebum *in vivo*. Also, it must be taken into consideration that many other factors are involved in *Malassezia*-related disease states and that the skin surface is a dynamic environment and many factors such as bacterial colonisation leading to the production of lipases which act in conjunction with sebaceous duct

lipases, and other external environmental factors contribute to changes in the chemical composition at the skin surface. What we have shown here is that sebum alone, in a high enough concentration is enough to stimulate growth of *M. furfur* and it may be more accurate to use sebum as a substrate instead of commercial lipid preparations when investigating pathogenicity. As mentioned previously, it is essential to provide a lipid substrate for *in vitro* growth of *M. furfur*. It has been shown that the fatty acid composition of *Pityrosporum* (*Malassezia*) *ovale* is dependent upon the particular fatty acids present in the medium [161,205] which means that the same strain, cultured with different lipid supplements may be biochemically different. Nazarro-Porro *et al* [161]suggested that fatty acids were not essential for growth but were likely to be important in membrane synthesis.

5.6.2 Growth of *M. furfur* in the presence of concentrated sebum and plucked anagen hair shafts

In a variation of the *M. furfur* viable growth study in the presence of dissected follicle components, the effect of the addition of concentrated sebum and other lipids to plucked anagen hair shafts was investigated. For both donors examined, viable yeast cell growth was observed in cultures containing concentrated sebum only or sebum plus dissected hair shaft component. The addition of squalene to dissected hair shaft components did not stimulate *M. furfur* growth. *M. furfur* was also observed not to be able to utilise squalene or oleic acid as a sole substrate for growth. The lack of *M. furfur* growth in oleic acid only containing cultures may be expected. Benham [19]

stated that *P. ovale* requires added oleic acid for growth. However, although Shifrine and Marr [205] found fatty acids essential for growth, they stated that oleic acid was unable to stimulate *P.ovale* growth when in a pure form. They speculated that the stimulation of *P.ovale* growth by oleic acid was due to the contaminating myristic and palmitic acids which were capable of supporting growth in the absence of oleic acid. This hypothesis was supported by Wilde and Stewart [236]. It is also possible that the carbon chain length of squalene is too large to be cleaved by *M. furfur* and does not induce the production of lipases.

In the case of oleic acid, the addition to hair shaft components also stimulated yeast cell growth. The addition of hair shaft components to concentrated sebum appeared to stimulate growth better than sebum alone suggesting that hairshaft factors and sebum factors both can play a role in growth and maintenance of *M. furfur* at the scalp surface. It is possible that *M. furfur* is not capable of breaking down oleic acid or squalene without the aid of lipase producing bacteria. As the plucked hair shafts were washed before use, many of the endogenous bacteria will have been removed. The minimal growth observed in the oleic acid plus hair culture may be due to residual bacterial activity. This study also shows that sebum contains many utilisable growth sources for *M. furfur*. The main difference is likely to be the fatty acid composition which is a consequence of bacterial lipase activity, the breakdown products of such activity being of a utilisable size for *M. furfur* yeasts. Because growth was not stimulated in the presence of oleic acid or squalene alone it raises an interesting point in that it shows that these 2 substrates will not induce lipase activity of *M. furfur*. Various studies on the lipase activity of *Malassezia* yeasts have been carried out. Ran *et al* [181]suggested that rapidly increasing lipase activities of *M.furfur* in the early

logarithmic growth phase of cultivation and accelerated growth in the presence of a lipase activator indicated the importance of lipase to hydrolyse nutrient fat for cell growth and development. I feel that the lipase activity is most likely to be substrate dependent in vitro and since a substantial proportion of the skin surface melee of lipids is squalene in the presence of bacterial breakdown products that are utilisable by *M. furfur* then I feel this supports the hypothesis of an *M. furfur* lipase redundancy *in vivo*, unless the bacterial populations are suppressed by drug treatment.

5.6.3 Growth of *M. furfur* on concentrated sebum-supplemented stripped stratum corneum samples

An interesting difference in models was also noted when it was observed that *M. furfur* growth was stimulated on SSC in the presence of 8% squalene. Growth in the squalene supplemented samples was much greater than in the sebum-supplemented samples. This I feel supports the above hypothesis that bacterial lipases are necessary to breakdown squalene and other lipids into a utilisable form for *M. furfur*. These skin biopsies by the D-squame method will remove surface bacteria and other skin commensals, which will therefore still be present and active after biopsy. We observed with SEM the presence of such bacteria. *Staphylococcus* species and *Propionibacteria* species will utilise lipids for growth, by the production of lipases. This also may suggest that *M. furfur* can also utilise keratinocytes as a growth stimulator or substrate, but only in the presence of a utilisable growth source. It would be interesting to compare the skin surface lipid composition of these supplemented

biopsies prior to and after growth of *M. furfur* to note the changes in fatty acid composition. Oleic acid-supplemented SSC sample s also stimulated yeast cell growth better than the hair-shaft supplemented oleic acid cultures. This suggests again that bacterial activity is more involved in the ssc model than the hair shaft model. This hypothesis could be examined by pretreating the biopsies with antibiotics which the bacterial population are likely to be susceptible to. Once the bacterial population has been suppressed the samples could be treated with lipid and then inoculated. No stimulation of growth would indicate the activity of bacterial lipases in the stimulation of growth observed in this study. When hair-follicle component- conditioned sebum was added to SSC model a further stimulation of growth was observed. This shows that perhaps that the incorporation of the 3 models has a synergistic effect on the growth of *M. furfur*, but that all are capable of acting as sole stimulators of *M. furfur* growth.

5.6.4 Growth of *M. furfur* on serial stripped stratum corneum samples

The serial stripping experiment did not show any major differences in stimulation of *M. furfur* growth. At all levels of the stratum corneum examined, variable *M. furfur* morphology was observed which is most likely to do with the pleomorphism of *M. furfur* rather than any different growth stimulators at various stratum corneum levels. An interesting phenomenon was the continuous, though low frequency, of hyphal filaments observed in control samples. Filaments were observed on corneocyte surfaces as well as burying underneath or through keratinized material. This may

indicate that there is a starvation switch which could be one mechanism involved in the Y-H transition as no filaments were observed on the surface strippings where there is likely to be the highest concentration of sebum lipids. Dorn and Roehnert [63]also suggested starvation as one possible mechanism of stimulating the Y-H transition when he observed filament production in a liquid starvation medium containing glycine. It may be expected that as growth penetrates deeper into the stratum corneum, there are less lipids present therefore hyphae form which scavenge for lipids on an intercellular and intracellular level. Tosti *et al* [218]speculated that filament production occurred in the lower 2/3 of the stratum corneum and was involved in the liberation of yeast cells or phialospores which could be returned to the surface by epidermal turnover to reinitiate the infectivity process. The lack of filaments in the sebum-supplemented strippings backs up the hypothesis that a starvation mechanism is involved. However, this brings about the question of whether lipid composition at the skin surface is involved in the pathogenicity of pityriasis versicolor, where high frequencies of filamentation are observed. More information is required on this subject. The observation of pits on corneocyte surfaces where yeast cells had been also suggests that the yeast cells produce substances which can breakdown keratinaceous material. As mentioned previously, there is no indication to date that *Malassezia* yeasts produce keratinases similar to those of the keratinophilic dermatophytes. This is definitely a point worthy of investigation. It is most likely necessary to have an understanding of what causes the coating to form in comparison to the actual surface lipidic coating of the yeast cells themselves. If the properties of the coating can be characterised then its role in pathogenicity may be elucidated.

5.7 CONCLUSIONS

From this study we can conclude that sebum at concentrated values associated with hair shafts and stratum corneum all may enhance the growth of *M. furfur*. Stratum corneum and hair shaft factors are most likely to be associated with lipids and fatty acids present and the action of the endogenous bacterial population.

CHAPTER6: GENERAL DISCUSSION

GENERAL DISCUSSION

Various *in vitro* models were used to assess growth and morphology of *Malassezia* yeasts. The morphological transformation from yeast growth to hyphal growth was also assessed under various conditions. Stripped stratum corneum samples supplemented with either 8.0% squalene or 1.5% cholesterol were observed to stimulate the yeast-hyphal transition in *M. furfur*, *M. sympodialis* and *M. slooffiae* isolates. It is proposed that squalene, a sebum component, may stimulate this transition in the presence of cutaneous bacterial flora by increasing the production of ergosterol, which plays an integral part in maintaining fungal membranes. Cholesterol, a secondary fungal membrane sterol, was also observed to stimulate the yeast-hyphal transition. It is proposed that the effect of membrane sterol content on the yeast-hyphal transition may cause a membrane vacuolation process by altered fluidity, similar to that observed from blastoconidia into the extending germtube in *C. albicans*. Gow *et al* [94] demonstrated a process of membrane vacuolation in apical expansion. As the cytoplasm migrates into the extending germtube, the parent cell becomes highly vacuolated. They also showed that cytoplasmic regeneration must occur before secondary germtubes and branches can be formed. Because high vacuolation will be observed in subapical compartments in *C. albicans*, it is thought that this explains why branch formation is sparse in *C. albicans*. This is an observation also made on the formation of *Malassezia* filaments. It is possible, therefore that a similar process of filament formation exists in *Malassezia* species.

Other evidence that altered sterol composition may effect hyphal growth is observed in the literature. Shimokowa *et al* [206]showed that *C. albicans* isolates

that were polyene-resistant mutants, accumulated 14-methyl sterols instead of ergosterol that prevented germ tube formation. This team showed that on analysis of revertants, sterol composition was normal and hyphal formation occurred, and they proposed an intrinsic rather that a fortuitous relationship between sterol composition and morphogenesis. Sadamori [196] have shown that ergosterol content of hyphal in *C. albicans* is 1.7 times higher that the content of *C. albican's* yeast cells. Therefore, a role exists for the involvement of sterol composition and filament formations in some fungal systems. Ergosterol may be required for dimorphic transition, or its depletion plus accumulations of other sterols may be lethal for filamentous growth.

Further experimental work could be carried out to test the hypothesis of the role of membrane sterols in the yeast hyphal transition in *Malassezia* yeasts and the mechanisms by which this occurs, It would be pertinent to examine the effect of altered sterol composition on *Malassezia* growth. This could be done by observing dimorphic ability of ergosterol-deficient mutants or in antibiotic resistant mutant s that accumulate 14-methyl sterols.

The stripped stratum corneum model could be supplemented with other membrane sterol precursors, as well as other sterols, to observe whether they have a similar effect on inducing this transition. For example, ergosterol samples as a lipid supplement on stripped stratum coreum samples could be examined to observe whether it simulated filamentous growth, as in the plant pathogen *Phymatotrichum ommivorum* [10] or a negative effect as observed in *C. albicans* [222]. Other precursors of ergosterol such as acetate and lanosterol could be examined by the same method.

Further parallels to processes involved in the yeast to hyphal transition in *C. albicans* were also apparent. Although it was shown that Dorn's filamentation broth stimulated filamentous growth in *Pityrosporum* (*Malassezia*) isolates when incubated at 29°C, magnesium sulphate appeared to be the most important media component in the stimulation of the yeast-hyphal transition in *Pityrosporum* isolates rather than glycine as proposed by Dorn and coworkers [63]. Removal of glycine, magnesium sulphate or ferrous sulphate prevented the transition taking place in the isolate examined, but magnesium sulphate was the only one observed to stimulate the yeast-hyphal transition on resubstitution to the remaining media components. In *C. albicans*, hyphal growth has been observed to be inhibited when grown in Mg-deficient media or in the presence of divalent ion chelators. Walker *et al* [226] also showed that a sharp peak in intracellular Mg concentration coincided with the onset of germtube formation. Taken into consideration, along with the work of Widra [233] and Gooday [90], these results suggest a central regulatory role for magnesium ions in *C. albicans* morphogenesis.

To reinforce the observations made in this study, similar studies could be carried out with magnesium ion and ferrous ion chelators to see if the yeast-hyphal transition is inhibited in *Malassezia* isolates which have a known filamentous capacity. As with *C. albicans*, this work has indicated that the yeast-hyphal transition in *Malassezia* yeasts is a multifactorial phenomenon with different internal mechanisms being activated by different exogenous factors such as divalent action availability and sterol and sterol precursor availability. Although only three species were observed to form filaments, a larger study of a wider variety of isolates would

have to be undertaken to establish the true pattern of hyphal growth over the seven species. According to the classification of Guého *et al* [97], only *M. furfur*, *M. globosa* and *M .obtusa* were observed to form filaments, although it was clearly stated that each species has to be examined carefully to see if this property is possessed. Between Gueho's work and the evidence highlighted here, we can conclude that the majority of *Malassezia* species may form filaments and the need to pool evidence and study a wider variety of isolates is highlighted.

Unlike *C. albicans*, where induction of filamentous growth *in vitro* can be used as a criterion for identifying this yeast, not all isolates testing for each species were able to form filaments, therefore in direct culture examination, other morphological criteria must be taken into consideration to differentiate between species by microscopy.

Using electron microscopy to assess *M. furfur* morphology under various conditions, a number of interesting observations are highlighted. Distinct collarettes could be clearly observed where the bud detached from the parent cell, indicative of repetitive budding from a single site. However, filaments were observed extending from various points of the parent cell surface. In particular, phialospore production was observed, where a filament extended in one direction whilst philospores were liberated from the opposite side of the parent cell. It is thought that the production of phialides may play a major role in the pathogenicity of pityriasis versicolor as the deeper penetration of the stratum corneum by the filaments can be balanced by the production of spores which can be returned to the stratum corneum surface by epidermal turnover to reinitiate the infection process. It is most likely to be observed

at different layers of the stratum corneum and not merely in the lower 2/3 as stated by Tosti *et al* [218]. It is most probably an important factor in the recurrence or relapse of patients with pityriasis versicolor.

In comparison, *C. albicans* has multiple budding sites but the yeast-hyphal transition is characterised by the bud elongation, or germ tube, process that is observed [166]. There is evidence that germ tube-like filamentation may occur in some *Malassezia* species. Gueho *et al* [97]indicated that *M. globosa* filaments are similar to these of *Candida albicans* in that they are formed at the origin of the bud, in comparison to *M. obtusa*, where filament production may occur at any point of origin on the mother cell surface, with a capacity for branching. Randjandiche [182]also shows that *P. ovale* (*M.furfur*) shows various methods of filament formation, with the formation of short (14μm) or long (19-35μm) filaments particular to the spherical *P. ovale* cells when isolated on Lowenstein or Kurung media supplemented with olive oil, and a fourth type characteristic of a germinative type similar to those of *C. albicans*. In this study, bud elongation was evident in some cases, but the requirements for qualifying as a germ tube is that the budding cell is not less that two and a half times the length of the parent cell, budding from a constricted base. However, the polymorphism of filaments observed in *P. ovale* by Randjandiche is similar to the observations made in this study [182].

In this study, a consistent role for hyphal production in seborrhoeic dermatitis and dandruff has not been shown. Although filament production in various isolates was achieved, there was no evidence to suggest that filament production exacerbated these disease states. Filaments may be isolated from lesional sites in

patients with seborrhoeic dermatitis and dandruff, but under the right conditions, *Malassezia* yeast cells are capable of causing these disease states without penetration of the stratum corneum by hyphal elements. Whether the yeast cells employ enzymatic mechanisms to invade the stratum corneum or whether damage to the stratum corneum is a prerequisite for invasion in these disease states is as yet unknown.

Another interesting morphological observation was the presence of an extracellular coating associated with the yeast cell surfaces when grown on stratum corneum samples supplemented with lipid, unsupplemented stratum corneum strippings, living skin equivalents, hairshaft components and sebum suspensions. In the latter two models, the inoculating suspensions had been prewashed in PBS containing 1.0% alcohol with an old solution to remove any surface lipids prior to addition to the experimental models. This coating was observed in the presence or absence of exogenous lipids in each model, which means that the coating observed in the electron micrographs of hair and stratum corneum samples are most likely formed by the organisms and are not merely derived from the lipid substrates. Such a coating may play a role in the pathogenesis of seborrhoeic dermatitis or dandruff. The most prominent example of extracellular coatings being involved in fungal pathogenicity is in the *C. albican's* model. Douglas [65] has clearly shown an extracellular proteinaceous material (EP) involved in the adhesion of *C. albicans* to host cells. This EP is formed from the fibrillar adhesive layer on the yeast cell surface. Other studies have indicated a positive correlation between *C. albican's* adherence and pathogenicity.

In *Malassezia* yeasts, adhesion properties have been demonstrated. Faergeman *et al* showed that adherence increased with time, increasing temperature and increasing inoculum size that was most likely due to receptor and enzyme systems that are time- and temperature-dependent [73]. A later study by Bergbrant and Faergemann [23] showed no indication of adhesion to stratum corneum cells being important in the pathogenesis of seborrhoeic dermatitis. In this study, stratum corneum cells from patients with seborrhoeic dermatitis showed lower numbers of adherent yeast cells in comparison to those observed on statum corneum cells from healthy individuals. However, there is evidence *in vivo* of an extracellular thin horny layer associated with clumps and chains of yeast cells and hyphae in cases of pityriasis versicolor [218]. This coating is most likely involved in adhesion of *Malassezia* cells to stratum corneum cells, keeping the yeast cells in contact with the surrounding environment. It may also provide a mechanism by which the yeast cells can invade host defence mechanisms. Studies by Mittag also provided evidence of an extracellular capsular-like layer associated with *Malassezia* yeast cell surfaces, similar to that observed in *C. albicans* [155,156]. Although Mittag suggested a lipidic nature to this coating, in the present study, this coating was observed to form under a number of experimental conditions. Various staining methods may be used to examine the nature of the coating before its exact composition can be determined. If, as the work of Zagari *et al* [245,246] indicates, *Malassezia* cells act in an allergenic capacity rather than a pathogenic capacity, then such a coating may be important in keeping allergenic cell surface components in contact with that stratum corneum surface, inducing disease. Until this coating has been characterised it will be difficult to assess whether or not its presence enhances the pathogenic activity of *Malassezia* yeasts.

Fibrin-like structures were also observed, which were formed under various conditions examined. These could be observed to attach yeast cell to yeast cell as well as yeast cell to corneocyte and hair shaft surfaces. Again, the most likely role for such structures is in adhesion to host surfaces. These structures may be a prerequisite for formation of the extracellular matrix discussed above, similar to the association between the *C. albicans* fibrillar layer and extracellular polymeric materials.

Considering the work of Rashid *et al* [183], the LSE model was adapted for use in the investigation of *M. furfur* colonisation of the stratum corneum. Destruction of the surfaces of the model after incubation with *M. furfur* yeast cells was similar to observation made *in vivo* on dandruff sufferers. *M. furfur* did not penetrate below the superficial layer of the stratum corneum of the skin models, unlike the dermatophytes in Rashid's study. This may be expected as deeper invasion of the statum corneum by *Malassezia* is normally only seen in cases of pityriasis versicolor, where the production of filaments aids deeper penetration. Filament production was not observed in this model that means that it did not contain the correct signal for stimulating hyphal production. However these models did not contain nutrients and signals capable of stimulating yeast cell growth as evidence by the increase in number of viable yeast cells with a three day incubation period on the skin model at 37°C. Because growth can be sustained on an LSE, it means that they can be utilised in the investigation of anti-*Malassezia* agents. Viable yeast cell growth can be measured on an LSE, and as they can be fixed and processed for S.E.M., LSEs can be used to observe the effect of antifungal drugs on yeast cell

morphology in the presence of keratinocytes. LSEs may therefore aid further research into the search for new target sites for antifungals.

In this study it was observed that *Malassezia* yeasts appear to express a preference for adherence and colonisation of the most lipid rich region of the hair shaft *i.e.* the nonemerged shaft, which lies in the infundibulum region of the hair follicle. This was shown by assessing the number of yeast cells that adhered to the difference portions of the hair shaft, when hairshaft components were added to PBS suspension of *M. furfur*. This suggests that a possible association of sebum components with hair shaft components provides a stable ecological niche for the growth of *M. furfur*.

In some hairshaft samples, mass destruction of the cuticular layer was observed when dissected anagen hair shafts were infected with a large inoculum of yeast cells on top of a floating filter. However, of all the hairshaft samples examined, this was observed to be an infrequent phenomenon. It is most likely that due to mechanical damage and the high number of yeast cells, the damage observed is circumstantial. In other instances, the yeast cells were observed to exist on the cuticular layer or damage exerted upon the hairshaft. Unlike the dermatophyte fungi, there is no evidence to suggest that *Malassezia* yeasts liberate extracellular keratinases. Hsu and Volz [111] have clearly demonstrated this phenomenon in hair invasion by the dermatophyte fungus, *Trichophyton terrestre* [111]. Complete digestion of human hair was demonstrated, due to lysis by enzymes liberated from *T. terrestre*. Dermatophyte fungi also develop appendages that aid the invasion of keratinaceous tissue. It is well documented that dermatophytes have distinct stages

in the invasion of human hair tissue. These stages consist of 1) Cuticle lifting by the production of enzymes and germlings which can penetrate between the cuticular layers [183]; 2) Cortical erosion by the production of frond-like mycelium [57]; 3) The production of penetrating organs, noted by Vanbreusegham, [220] and 4) Colonisation of the medulla [13]. Rashid *et al* [183]demonstrated similar modes of invasion on *in vitro* cultured human hair follicles as are observed *in vivo*. His work supported the use of such *in vitro* model in the examination of dermatophyte infection. In this study, I have shown that this model can be adapted to make observations on other cutaneous organisms and pathogens.

In some instances, *Malassezia* filaments were observed that showed no evidence of penetration beneath the cuticular surfaces. It is probably unnecessary to investigate penetration of *Malassezia* yeasts under the cuticular layer any further as there is no evidence to suggest that *Malassezia* yeasts require to invade the hairshaft any further that the surface layer to cause infection. Penetration occurring under this layer in infection is most likely to do with the state and condition and subsequent colonisation by *Malassezia* species. It is probably more important to study the location and proliferation of *Malassezia* yeasts in the external follicular environment of the hairshaft.

Growth of *M. furfur* was also stimulated by hair shaft components or their soluble factors *in vitro*. This stimulation of growth appeared to be dependent on hairwashing patterns of the donors and the relative "greasiness" of the hair. Shaft components from donors who washed their hair once to twice daily were not capable of stimulating yeast cell growth. As time intervals between hair washes increased,

shaft components, in particular non emerged shaft components, were capable of stimulating viable growth of *M. furfur* and *M. sympodialis* after three days incubation at 29°C.

Stimulation of growth by the emerged shaft components and the unsheathed shaft components was also observed to increase with increasing time interval between donor hairwashes. This also adds weight to the belief that *Malassezia* yeast will show a predilection for the most lipid-rich environments on the host. It also indicates a role for lipid-breaking bacteria in the survival of *Malassezia* yeasts in the follicular habitat. Lipids must be produced to provide a substrate for *Malassezia* growth, and the longer left between hairwashes, the greater time for the endogenous follicular bacteria to exert lipase activity, breaking down the follicular lipids into forms more useful to *Malassezia* yeasts. It is most likely that follicular shaft colonisation is indicative of the yeast cell populations observed at the stratum corneum. *Malassezia* cells may be transported towards the stratum corneum surface with hair growth and spread across the skin by sebum dispersal. As most of the follicular microflora appears to be removed by hair washing regime, it is most likely that recolonisation of the follicle occurs through agitation at the skin or scalp surface, pushing organisms back down the follicular orifice. This is most likely to occur in pathogenic and non-pathogenic states.

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APPENDIX 1

Dixon's agar composition

Ingredients (to make up 500ml agar)

Oxoid malt extract 18g
Oxoid mycological peptone3g
Oxoid purified agar 5g
Sigma ox-bile 10g
Tween 40 5ml
Glycerol 1ml
Oleic acid 1ml
Distilled water 483ml

Sorrenson's Buffer composition pH 5.6

M/15 Sodium phosphate dibasic = 9.465g (Na_2HPO_4) per litre of distilled water
M/15 Potassium acid phosphate= 9.07g (KH_2PO_4) per litre of distilled water

5.0 cm^3	M/15 Sodium phosphate dibasic
95.0 cm^3	M/15 Potassium acid phosphate

APPENDIX 2 -ABBREVIATIONS USED

SSC:	STRIPPED STRATUM CORNEUM
IRS:	INNER ROOT SHEATH
ORS:	OUTER ROOT SHEATH
MEM	MINIMAL ESSENTIAL MEDIUM
DSA:	DIAGNOSTIC SENSITIVITY AGAR
PBS:	PHOSPHATE BUFFERED SALINE
FCS:	FOETAL CALF SERUM
TBS:	TRIS BUFFERED SALINE