POTENTIAL THERAPEUTIC APPLICATION OF FUNGAL FILAMENTS IN WOUND MANAGEMENT

PAUL F. HAMLYN[1] & RICHARD J. SCHMIDT[2]

[1]*British Textile Technology Group, Shirley Technology Centre, Didsbury, Manchester M20 2RB*
[2]*Welsh School of Pharmacy, UWCC, Redwood Building, King Edward VII Avenue, Cardiff CF1 3XF*

Introduction

The use of fungal materials as styptics in folk medicine has been documented (Baker, 1989; Vaidya & Rabba, 1993) as has the use of mouldy bread, jam, etc. and, more recently, live mycelial pads for the prevention and treatment of wound infections – efficacy apparently being related to antibiotic (patulin rather than penicillin) content (Wainwright, 1989; Wainwright *et al.*, 1992).

With the availability of haemostats (styptics) based on calcium alginate (a seaweed-derived polysaccharide), and the preferred use of systemic rather than local antibiotic therapy, it would appear that there is no place for fungal materials in modern wound management. It may seem curious, therefore, that novel fungal materials with the potential to be used in wound management are currently being developed.

The major components of fungal cell walls are polysaccharides which typically account for 80–90% of the dry weight, the remainder consisting of protein and lipids (Bartnicki-Garcia, 1968). Chitin is present in the majority of fungi as one of the skeletal polysaccharides responsible for maintaining the rigidity and shape of the cell wall. It is a β-1, 4-linked linear homopolymer of N-acetyl-D-glucosamine similar to cellulose but with an acetamido group rather than an hydroxyl at C-2. Chitin is not the only structural polysaccharide present in fungal cell walls. The alkali-insoluble cell wall residue of ascomycetes and basidiomycetes has been reported to consist of a branched β-1,3-/β-1,6- glucan together with chitin (Rosenberger, 1976). In zygomycetes in particular, the chitin may be partially deacetylated (the fully deacetylated material being known as chitosan). The sugars fucose, galactose, mannose and xylose have been identified as minor components in hydrolysates of cell walls from various fungal species whilst galactosamine has also been identified in the cell walls of several fungi, though usually accounting for only a few percent of the total monomers. Furthermore, the chitin content varies between species and even growth forms (e.g. vegetative mycelium, sporangiophores) and can be greatly influenced by the composition of the growth medium and the conditions of culture.

Chitin is not unique to fungi, also being found in the shells of crustaceans and in the exoskeletons of insects (Austin *et al.*, 1981). Indeed, chitin is currently obtained commercially from crab- and prawn- shells which are waste products of the food industry.

Scientific studies on the use of chitin in wound healing were first reported in the 1970s (Balassa, 1972; Balassa & Prudden, 1978). In rats, the bursting strengths of experimental wounds treated with chitin of crustacean or fungal origin, were found to have increased by up to 60% relative to controls. Since then, further evidence has been obtained to suggest that chitin has wound healing acceleration properties although the mechanism of action is still largely unknown (Olsen *et al.*, 1989).

Filamentous fungi are an attractive source of chitin for medical application because specific products can be manufactured under standard conditions and the chitin is produced in a fibrous form so that only relatively cheap chemical treatments are required during processing. This article summarises a programme of work carried out at BTTG and the Welsh School of Pharmacy to investigate the potential application of fungal filaments in wound management.

Experimental Materials

At BTTG a range of filamentous materials was prepared containing from about 20% (w/w) up to

90% (w/w) chitin[1] (Table 1). In addition to mycelia from *Fusarium graminearum, Rhizomucor miehei, Rhizopus oryzae* and *Mucor mucedo*, sporangiophores of *Phycomyces blakesleeanus* and *Agaricus bisporus* stipe (a waste product from the cultivated mushroom industry) were also included in these investigations. All the products had been treated with 2M NaOH at 100°C for 1 h to remove protein and other alkali-soluble components. Further details about the fungal culture conditions and the preparation of the filamentous materials are given in Chung *et al.* (1994).

Cell Proliferation Studies

Mammalian cell culture systems were used for evaluating bioactivity. This work was carried out at the Welsh School of Pharmacy where comparative bioassay systems have been developed to assess and compare materials used in wound management (Turner *et al.*, 1989; Schmidt *et al.*, 1989; Schmidt *et al.*, 1993). The objective of these studies was to establish whether any of the fungal materials can affect the rate of proliferation of fibroblasts, the cell type responsible for laying down new (granulation) tissue in a healing wound, and therefore whether the materials had the potential to increase the rate of wound healing. A second objective was to determine whether there is a correlation between the chitin content of the materials and their effects on fibroblast proliferation.

At high concentrations, the purified fungal preparations had anti-proliferant activity. This

[1] It should be noted that the assay method we used was not able to differentiate chitin and chitosan. The assumption made for the purposes of calculation was, therefore, that only chitin was present. The fungal materials are more appropriately described as containing 'chitin/chitosan'.

Table 1. Effect of fungal materials (0.01% w/v) on the proliferation of murine L929 fibroblasts in culture after 6 days

Organism	Growth form	Chitin[a]%	Cell no. × 10^4 cells ml^{-1} Mean ± S.D. (n = 3)	% Change
Control	–	–	138.95 ± 4.02	–
Fusarium graminearum	mycelium	19	135.55 ± 2.75	−2
Agaricus bisporus	stipe	42	144.00 ± 4.41	+4
Rhizomucor miehei	mycelium	70	154.94 ± 4.86*	+11
Control	–	–	152.17 ± 7.34	–
Rhizopus oryzae	mycelium	72	171.34 ± 1.21*	+13
Phycomyces blakesleeanus	sporangiophores	91[b]	173.79 ± 2.16*	+14
		94[c]	178.78 ± 6.30*	+17

% Change: % Change in cell yield relative to the control
* $p < 0.05$ compared to the control (Student's t-test)
[a] Estimated chitin content. Some of the chitin could be deacetylated.
[b] Unbleached.
[c] Bleached.
Results taken from Schmidt *et al.*, 1994

Table 2. Effect of prepared sporangiophores (0.05% w/v) from Phycomyces blakesleeanus *on the proliferation of human F1000 fibroblasts in culture.*

	Day 3		Day 6		Day 9		Day 13	
	Cell no. x10^4 cells ml^{-1} Mean ± S.D. (n = 3)	% Change	Cell no. x10^4 cells ml^{-1} Mean ± S.D. (n = 3)	% Change	Cell no. x10^4 cells ml^{-1} Mean ± S.D. (n = 3)	% Change	Cell no. x10^4 cells ml^{-1} Mean ± S.D. (n = 3)	% Change
Control	14.0 ± 0.50	–	16.93 ± 0.90	–	20.11 ± 0.74	–	24.09 ± 0.23	–
P.blakesleeanus	14.49 ± 0.66	+3	20.22 ± 1.59*	+18	24.53 ± 1.31*	+22	34.22 ± 2.10	+42

% Change: % Change in cell yield relative to the control
* $p < 0.05$ compared to the control (Student's t-test)
Results taken from Chung *et al.*, 1994

did not correlate with chitin content and may have been the result of a direct chitosan/plasma membrane interaction (Chung *et al.*, 1993). At lower concentrations, pro-proliferant effects which appeared to correlate with chitin content were observed. These effects were most marked with material derived from sporangiophores of *Phycomyces blakesleeanus* which had the highest chitin content of all the fungal preparations examined (Tables 1 & 2). Material derived from sporangiophores of *P. blakesleeanus* or mycelium of *Mucor mucedo* also exhibited cell attractant and cell binding properties in fibroblast cultures (results not shown), effects that could conceivably assist movement and attachment of fibroblasts in the healing wound. (Fibroblasts are responsible for laying down collagen in the wound and hence rebuilding tissue.)

In order to explain the pro-proliferant effects of the fungal materials, a mechanism involving auto-oxidatively generated hydrogen peroxide has been proposed (Chung *et al.*, 1993; Schmidt *et al.*, 1994). Auto-oxidation is the process of spontaneous oxidative degradation in air; this process has long been known to lead to hydrogen peroxide formation and requires the presence of traces of transition metal ions (such as iron or copper) to proceed. Hydrogen peroxide acts as a fibroblast cell proliferant at the very low concentrations generated by auto-oxidation of the materials in the culture medium (Burdon *et al.*, 1989; Schmidt *et al.*, 1992). Addition of catalase (an enzyme which specifically destroys hydrogen peroxide) to the culture media completely abolishes the pro-proliferant effects of the fungal materials. It was also found that the hydrogen peroxide-generating capacity did not correlate with chitin content in these fungal materials. Our more recent work has demonstrated that hydrogen peroxide generating capacity appears to be a property of chitosan rather than chitin, at least in crustacean-derived materials (Chung *et al.*, 1993).

Fabrication

It is envisaged that two basic types of dressing could be directly manufactured from fungal filaments: an absorbent freeze-dried pad for deeper wounds and a thin wet-laid surface dressing (Hamlyn, 1991). The two types of product could be used in conjunction with other materials to form a multi-layered structure (Sagar *et al.*, 1990).

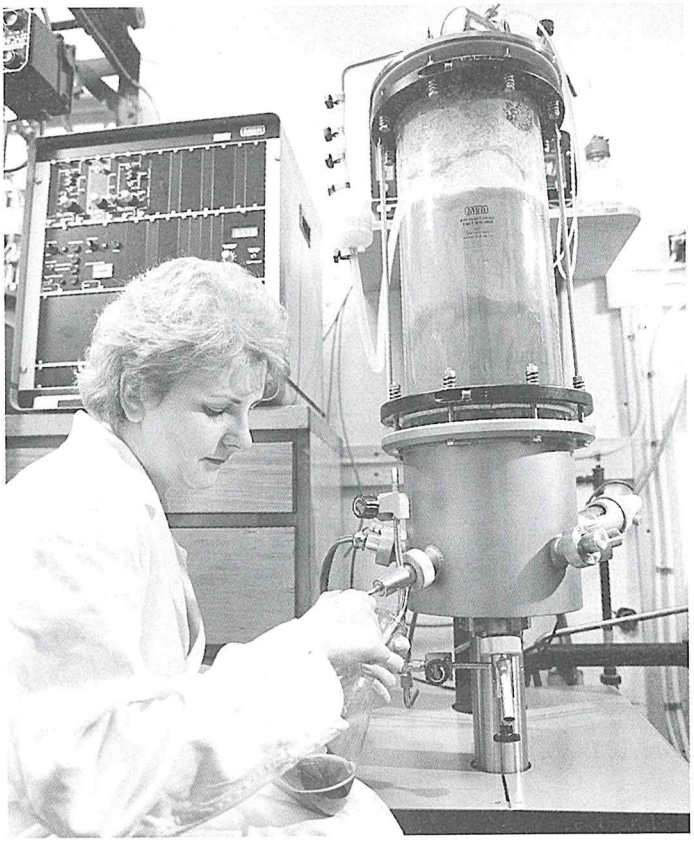

Fig 1 Pilot scale 20 litre bioreactor.

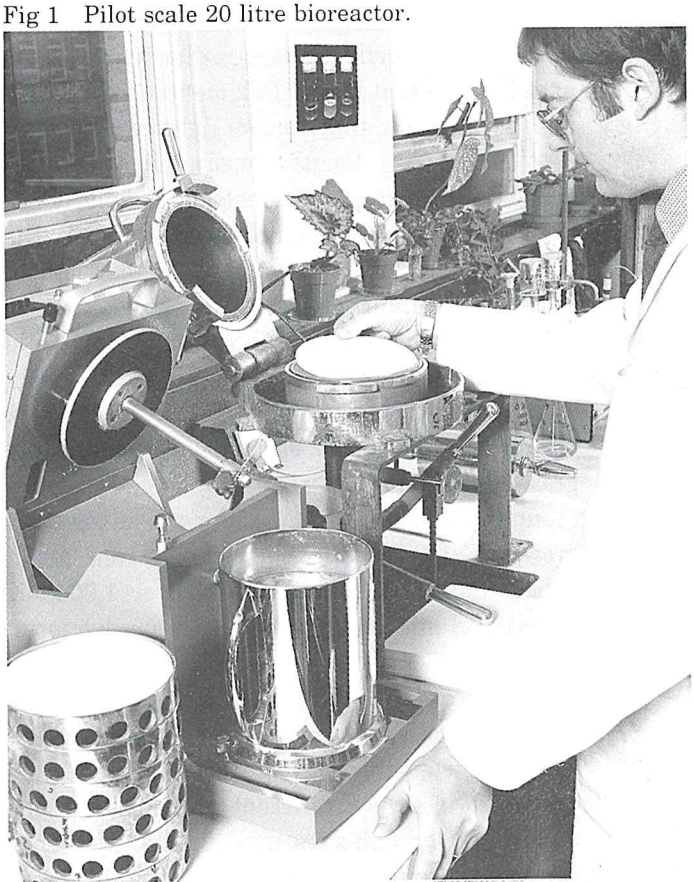

Fig 2 Preparation of wet-laid mats from fungal filaments using a British Standard Sheet Paper-making machine.

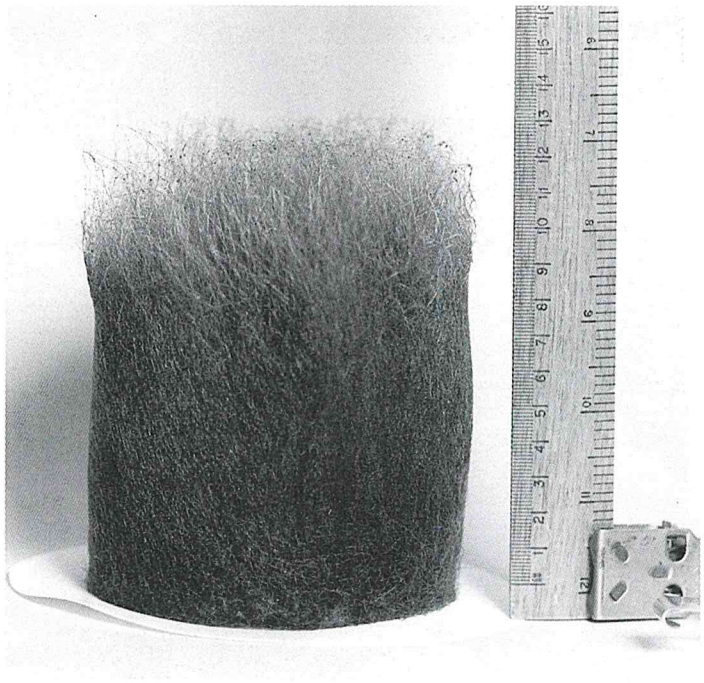

Fig 3 Sporangiophores of *Phycomyces blakesleeanus* produced after 10 days.

In comparison with conventional natural fibres, fungal filaments have the advantage that the biomass is available in days rather than months and that different kinds of filamentous structures are available, ranging from straight fibres several centimetres in length (sporangiophores) to branched microscopic filaments (mycelium). Fungal filaments are also hollow, leading to the possiblity of their development as carriers of micro-encapsulated drugs.

Sporangiophores are only obtained on solid substrates similar to those employed for mushroom cultivation, whereas vegetative mycelium can be produced most economically in a liquid growth medium inside a bioreactor (Fig 1). From a commercial point of view the technology for growing a fungus in a bioreactor is well established. Industrial bioreactors of over 1,000,000 litres in volume are now used for the production of biomass.

After growing a fungus in a bioreactor, the resulting broth contains very fine branched filaments which, after suitable processing, can be fabricated in several different ways. The broth is first passed through a fine mesh filter and the solid material thoroughly washed with water to remove residual medium. Depending on the end-use, alkali treatment can be employed to remove proteins and other impurities and the filaments can be bleached to give a white product. Such bleaching can, however, modify the bioactivity of the material (Schmidt *et al.*, 1994). The treated filaments are again thoroughly washed and finally re-suspended in water.

At this stage the filaments can be wet-laid, either on their own or mixed with conventional fibres such as wood pulp and polyester using normal laboratory paper-making equipment (Fig 2). When mixed with other fibres, the resulting composite mats are coherent but paper-like. On their own, the microfungal filaments are very brittle. However, these can be plasticised (for instance with glycerol) to form flexible, membrane-like materials. Another type of novel material can be made by freeze-drying a thick slurry of the filaments to produce an absorbent pad. In the case of sporangiophores (Fig 3), attempts to spin a yarn from these long straight filaments have, so far, proved unsuccessful because of their brittle nature in the dried state. Sporangiophores suffer from the dual drawback of having both low tensile strength and low breaking extension in comparison with conventional textile fibres (Table 3). Therefore, it has only been possible to fabricate these filaments by the methods already described after they have been cut into suitable short lengths to aid dispersion.

Following alkali treatment and bleaching,

Table 3. Tensile properties of sporangiophores from Phycomyces blakesleeanus *and some conventional textile fibres*

Fibre	Tenacity (cN/tex)	Breaking extension (%)
Sporangiophores (*P.blakesleeanus*)	7*	3*
Cotton	19–45	6–9
Wool	11–14	30–43
Flax	57	3
Jute	31–70	2
Viscose rayon	7–27	16–27
Casein	10	63

Tenacity: The strength of textile fibres is referred to as their tenacity determined by measuring the force required to break the fibre.
Breaking Extension: Measure of the extension of the fibre before failure.
* Mean of 20 determinations on individual filaments. The sporangiophores were conditioned at 65% Relative Humidity prior to testing.
Results taken from Hamlyn, 1991.

highly absorbent products can be made from the sporangiophores. The ultrastructure of these novel fungal products as revealed by the scanning electron microscope is shown in Figs 4 & 5.

Conclusions

The next generation of wound management products are expected to participate 'actively' in the wound healing process by releasing stimulatory molecules to the wound surface (Turner, 1986). These products are being developed, in particular, for the treatment of chronic wounds such as leg ulcers and bed sores which can take many months to heal by traditional means, thereby placing a considerable burden on hospital and nursing resources.

The results of the tissue culture studies reviewed in this article have been encouraging, indicating that fungal filaments exhibit biological activities (cell attractant, cell-binding, and pro-proliferant) of relevance to the wound healing process and that the pro-proliferant activity may be a feature of the chitosan present in the filaments, and more specifically the outcome of hydrogen peroxide generation by auto-oxidation of the fungal filaments. If these fungal materials were developed for use in wound management, it is conceivable that they would promote the growth of fibroblasts into the wound cavity and provide a matrix for their anchorage. This would lead to a more rapid deposition of new collagen and hence granulation tissue.

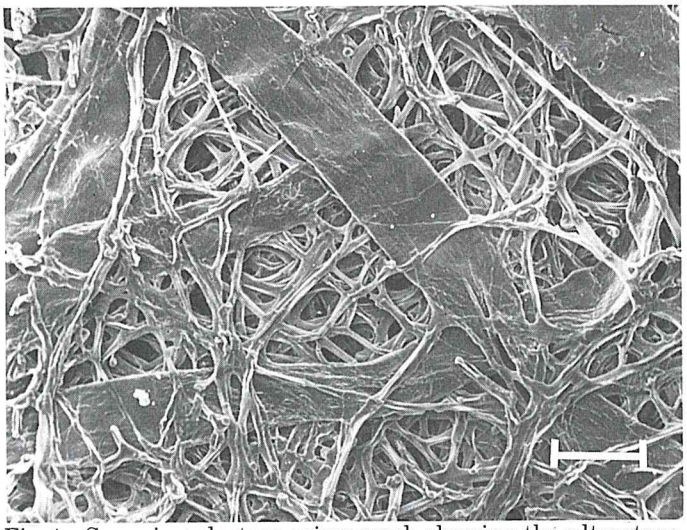

Fig 4 Scanning electron micrograph showing the ultrastructure of a composite mat composed of fungal and wood pulp fibres. Bar = 36μm.

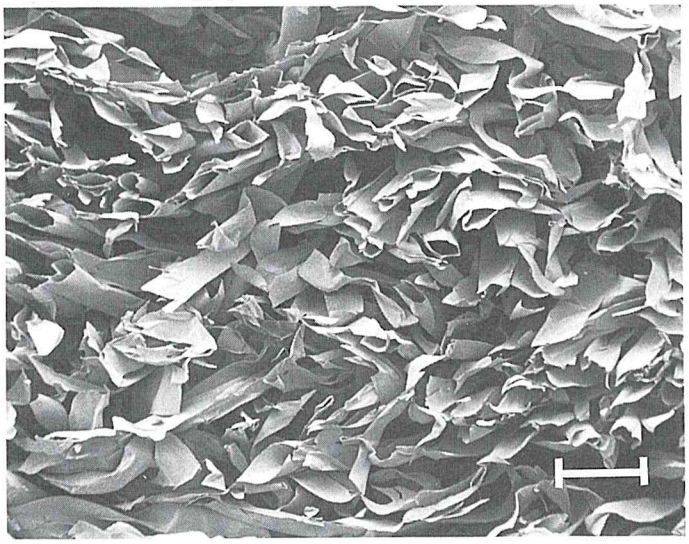

Fig 5 Scanning electron micrograph illustrating the cross-section of a pad composed of sporangiophores. Note the hollow nature of these filaments. Bar = 250μm.

Acknowledgements

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BOOK REVIEW

Biotechnology in Plant Disease Control
edited by I. Chet (1993 Wiley-Liss, New York). Pp. 373, hard back, ISBN 0-471-56084-7 Price US $99.95.

This book is full of excellent essays on the use of biotechnology in plant disease control. Despite the quotation from Einstein that imagination is more important than knowledge, readers will find that this very thorough manual provides plenty of information on the far reaching developments that biotechnology is bringing to the professional plant pathologist.

As well as the overview of the impact of biotechnology provided by Logemann and Schell and the specific case of the biocontrol of take-all disease of wheat discussed by Hemming and Houghton, there are comprehensive sections on molecular genetics in disease control, the introduction of disease resistance into plants and the application of biotechnology for improving biological control. While all of these sections provide much of interest to the general reader, I found that several of the chapters on the introduction of disease resistance into plants were especially stimulating, particularly that by J.P. Métraux and I. Raskin on the role of phenolics in plant disease resistance. Several of the chapters in the last section give valuable advice on the detection and diagnosis of plant pathogens.

This is a very ambitious book on an extremely wide topic that succeeds in its aim of reviewing this fast moving field where imagination is not a limiting factor.

Roland Fox