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Growth and viability of mycelial fragments of white-rot fungi on some hydrogels

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The viability of mycelial fragments of *Trametes versicolor* and *Irpex lacteus* and their growth on selected hydrogels are described. The size of mycelial fragments of the fungi did not significantly influence their viability. Alginate hydrogel films supported fungal growth better than agarose, carrageenan, chitosan and gelatin films, and had the highest mechanical strength but were less hydrophilic than the other hydrogels. All commercial alginates that were tested supported aseptic growth of fungal fragments without prior sterilization of the hydrogel solution. The viability of mycelial fragments in the hydrogel solutions was higher for some commercial alginates than that in laboratory grade alginate. The mechanical strength and hydrophilicity of hydrogels from alginate type Sobalg FD 155 and Meer HV were comparable to that of laboratory grade alginate. Sterilization and pH of the alginate hydrogel did not significantly influence the growth of *T. versicolor* mycelial fragments but affected the growth of *I. lacteus*. Concentrations of alginate in the range of 1–2% in the hydrogel did not affect the growth of entrapped mycelial fragments of these fungi.

Keywords: fungal inocula; mycelial fragments; commercial hydrogels

Introduction

The development of fungal inocula of uniform quality and resistance to competition by indigenous microbes is essential for such applications of fungi in the environment as biological control, bioremediation of contaminated soils, and inoculation of soils with mycorrhizal fungi. Encapsulation or immobilization of microbial biomass on a carrier may improve cell survival and provide a means of establishment of cells introduced into a site by protecting cells from various environmental stresses and by providing a stable microenvironment [2]. Formulation is often of paramount importance for efficiency of fungal inocula. A variety of natural, plant- or soil-derived materials such as peat, granular vermiculite mixtures, grains, wood chips, wheat straw, corn cobs etc have been tested as carriers of microbial biomass [2,5,6,11,16,19]. Lately alginate and other hydrogel granules have gained popularity since inoculant consistency and effectiveness may be enhanced due to the defined nature of these carriers [9,10,20].

Many microbial species have been encapsulated successfully in various matrices. However, encapsulation of microorganisms in a hydrogel-matrix for environmental release is still in the early stages of development [2]. Most reports on the use of hydrogels as immobilization materials have been based upon experiments with uncharacterized samples [12], despite the fact that physical and mechanical properties of hydrogels depend on the hydrogel source and can critically alter the viability of microbial propagules [4,12,18].

Fungal spores, due to their long-lasting viability, were preferentially used as propagules for encapsulation [1,3]. Not all fungi, however, sporulate readily and separation of spores from the mycelium is often difficult and a waste of valuable fungal biomass. In some studies encapsulation of mycelial fragments was reported [14,21], but the effects of fragmentation on the production of viable propagules and the viability of mycelial fragments were not investigated.

Recently, we reported on the development of novel fungal inocula for bioaugmentation of soils contaminated with hazardous organic compounds [7,8]. The inocula were in the form of pelleted solid substrates coated with a hydrogel suspension of fungal propagules and incubated until overgrown with a dense mycelium of selected white-rot fungi. Because of their lignin-degrading or wood-rotting enzymes, white-rot fungi have been reported to degrade and mineralize a wide variety of recalcitrant organic pollutants such as DDT and other pesticides, polyaromatic hydrocarbons, polychlorinated biphenyls, dioxins, wood preservatives such as pentachlorophenol, and conventional explosives such as TNT [17].

In this paper the viability of mycelial fragments of two white-rot fungi, *Trametes versicolor* and *Irpex lacteus*, was assessed. Some commercial hydrogels that could be used as fungal carriers or for coating the solid pelleted substrates as microbial carriers were tested.

Materials and methods

Organisms

Irpex lacteus (Mad-517, ATCC 11245) and *Trametes versicolor* (MD-277) were obtained from the Center for Forest Mycology Research, Forest Products Laboratory, Madison,

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WI, USA. The fungi were grown and maintained on 2% potato-dextrose agar (PDA) (Sigma, St Louis, MO, USA).

Materials

Technical grade Wego sodium alginate was obtained from Wego Chemical & Mineral Corp, Great Neck, NY, USA. Danisco sodium alginate (Sobalg FD 155) was obtained from Danisco Ingredients USA Inc, New Century, KS, USA. Sodium alginates (Meer HV and Meer LV) were obtained from Meer Corporation, North Bergen, NJ, USA. Commercial alginate samples (Kelgin XL, Keltex, and Kelgin MV) were obtained from Kelco, San Diego, CA, USA. Laboratory grade sodium alginate was purchased from Sigma.

Medium and culture conditions

Fungi were grown in 300-ml Erlenmeyer flasks containing 60 ml of growth medium (2% glucose, 2% malt extract, and 0.1% Tween 80) on a rotary shaker at 250 rpm and 30°C for 3–10 days to form mycelial pellets. The growth medium was inoculated with 6 ml of fungal fragments obtained from fragmentation of mycelial pellets, as described below.

Fragmentation of fungal mycelia

Mycelial fragments were used to study the influence of culture age and fragmentation time on fragment viability. *T. versicolor* and *I. lacteus* mycelial pellets were separated by decantation of growth medium, washed with 10 mm Na-tartrate (pH 6) and resuspended in 60 ml of the same buffer. Washed fungal pellets (3, 5, 7, and 10-day-old cultures) were fragmented two, four, six, eight, 11 and 14 times for 5 s at 22 000 rpm in a laboratory blender.

Mycelial fragments for inoculation of growth medium and hydrogels were prepared by fragmentation (six times for 5 s) of mycelial pellets from 4-day-old shake cultures.

The dry weight of mycelial fragments in the suspension was determined by collecting mycelial fragments on preweighed Whatman No. 1 filter paper, and weighing them after drying them at 60°C for 24 h.

Separation of mycelial fragments by size

Mycelial fragments for viability studies were separated by size to obtain a suspension of small single-hyphae fragments (A); a suspension composed of A and pieces of loose mycelium (B); and a suspension composed of larger and compact mycelium structures (C). Suspension A was obtained as supernatant after 20 s (*T. versicolor*) and 10 s (*I. lacteus*) centrifugation of mycelial fragments at $780 \times g$. Suspension B was obtained after 30 s (*T. versicolor*) and 20 s (*I. lacteus*) centrifugation. Suspension C (*I. lacteus* and *T. versicolor*) was obtained after five consecutive steps of 15-s centrifugation of mycelial fragments and washing of the pellet with 10 mm Na-tartrate (pH 6). The final pellet was resuspended in 60 ml of the same buffer. The number of mycelial fragments in suspensions A, B, and C was determined by counting with a haemacytometer. Mycelial fragments were stained with 0.3% aqueous methylene blue and microphotographed with a Wild Heerbrugg M4OD Photomakroskop.

Viability of mycelial fragments

Serial dilutions of mycelial fragments in 10 mM Na-tartrate (pH 6), were prepared. Aliquots (0.2 ml) were spread on PDA. Fungal colonies from mycelial fragments were counted after 18 h of incubation at 30°C, and populations were reported as colony-forming units (CFU).

Hydrogel film preparation

Agarose (Difco) and gelatin (Sigma) solutions were autoclaved for 10 min at 120°C prior to use. Autoclaving was also used to prepare sterile alginate hydrogel solutions. Hydrogel films were then prepared by spreading 5 ml of hot hydrogel solutions over glass Petri dishes.

Alginate, k-carrageenan (Sigma), and chitosan (Sigma) hydrogel films were prepared by spreading 5 ml of the hydrogel solution over glass Petri dishes followed by gelation with 5% $CaCl_2$, 5% KCl, and 5% Na-polyphosphate, respectively. The hydrogel solution was sprayed three times with the gelling agent and the hydrogel film was allowed to form for 3 min for alginate and carrageenan and 30 min for chitosan. To complete gelling, 5–10 ml of the gelling agent (1% $CaCl_2$ for alginate) was carefully poured into the dish, and incubated for 17 min for alginate and carrageenan, and 2 h for chitosan. Excess gelling agent was removed by washing the films twice with sterile deionized water.

To grow fungi on hydrogel films, hydrogel solutions containing 2% glucose and 2% yeast extract were used. When preparing alginate, carrageenan, and chitosan films, the gelling agent solutions and deionized water for film washing were also supplemented with 2% glucose and 2% yeast extract to prevent nutrients from being rinsed from the hydrogel. Enriched hydrogel films were inoculated by spreading 0.2 ml of a fungal fragment suspension, prepared as described above, over the film. The pH of the alginate hydrogel was adjusted by the addition of NaOH and acetic acid to the alginate solution.

Fungal biomass recovery

Fungal biomass grown on agarose, carrageenan, or gelatin films was determined by selectively dissolving the hydrogel by microwaving it [15]. The melted hydrogel and fungal mycelium were vacuum filtered and the fungal biomass was collected on preweighed Whatman No. 1 filter paper, rinsed with deionized water, dried for 24 h at 60°C, and weighed.

Fungal biomass grown on alginate and chitosan was retrieved by dissolving the hydrogel with 0.05 M Na_2CO_3, 0.1 M citric acid solution, pH 7 [13], and 0.5% acetic acid solution, respectively, for 15 min on a tumbler-shaker. Fungal biomass was collected on a preweighed 45-μm pore size cellulose acetate filter (Cole-Parmer, Niles, IL, USA) by vacuum filtration, and dry biomass was determined as described above.

Viability of mycelial fragments in alginate hydrogels

Solutions of commercial alginates were sterilized as described above and inoculated with 10% of *T. versicolor* and *I. lacteus* mycelial fragment suspensions, prepared as described above. The concentrations for Wego, Danisco, Meer HV, Meer LV, Kelgin XL, Keltex, Kelgin MV, and Sigma alginate solutions were 1.5%, 1%, 1%, 2%, 2%, 1.5%, 1.5% and 1%, respectively. Hydrogel films from

these alginate solutions had a strength of approximately 0.5 N. Before inoculation the pH of alginate solutions was aseptically adjusted to pH 6.5. Alginate solutions were incubated at 6°C or 24°C in triplicate. CFUs were determined shortly after inoculation and after 2 days of incubation as described above.

Microbial contamination of commercial alginate hydrogels

Ten- and 100-fold dilutions of 5% solutions of commercial alginates in sterile, deionized water were prepared. Dilutions (0.2 ml) were spread on PDA or Luria-Bertani-agar (LBA: 10 g L^{-1} bactopeptone, 5 g L^{-1} yeast extract, 2.5 g L^{-1} NaCl, 15 g L^{-1} agar, pH 7.2) for determination of fungal and bacterial contaminants, respectively. Plates were incubated at 24°C (fungi) or 30°C (bacteria) until colonies appeared, CFUs were then counted.

Mechanical strength of hydrogel film

Hydrogel films, obtained as described above, were cut in 5-mm-wide strips. The mechanical strength of the film was measured with a force gauge (spring scale). The peak force at the moment of strip rupture was measured and expressed in Newtons (N).

Measurement of equilibrium moisture content

Hydrogel films (1%) were placed in closed chambers, partly filled with saturated salt solutions to maintain constant relative humidity [23], and incubated at 30°C. After 15 days of equilibration, the moisture content was determined by weighing the strips before and after drying at 60°C for 24 h.

Results

As shown in Table 1, the size of mycelial fragments of 4-day-old *T. versicolor* and *I. lacteus* cultures did not significantly influence the viability ratio. Viability ratio was defined as CFUs per total number of fragments counted using microscope and haemacytometer. The viability ratio of small single-hyphae fragments (suspension type A) was not significantly different from the viability ratio of much larger and compact mycelial structures (suspension type C) with average diameters of 0.25–0.8 mm and 0.1–0.5 mm for *T. versicolor* and *I. lacteus*, respectively. Viability ratios higher than 1 for *I. lacteus* indicate the presence of small but viable hyphae fragments that were not observed under the microscope. The single-hyphae fragments (suspension type A) of *I. lacteus* and dense mycelial structures (suspension type C) of *T. versicolor* are shown in Figure 1.

The effect of culture age and time of fragmentation on viability of mycelial fragments is presented in Figure 2.

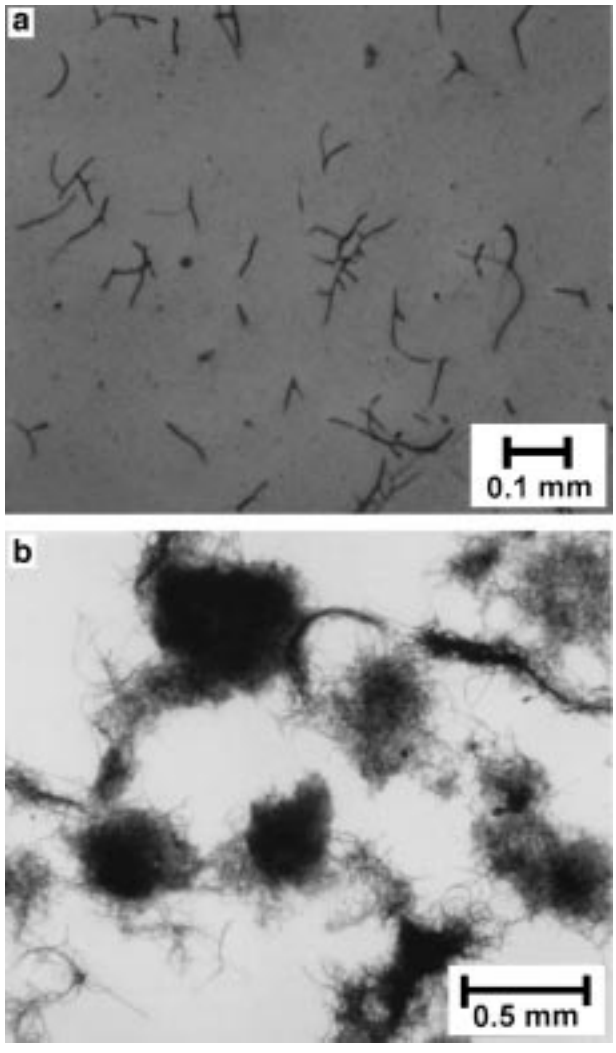

Figure 1 (a) Single-hyphae fragments of 4-day-old *I. lacteus* culture. (b) Compact mycelial structures of 4-day-old *T. versicolor* culture.

The fragments from 3-day and 10-day-old cultures of *T. versicolor* and from 7-day and 10-day-old cultures of *I. lacteus* had the highest viability. The viability ratio of fragments tended to decrease with increasing time of fragmentation (Figure 2a and c), while the total number of viable fragments in suspension tended to increase (Figure 2b and d).

Figure 3 presents the growth of mycelium fragments of *T. versicolor* and *I. lacteus* on alginate, agarose, carrageenan, chitosan, and gelatin hydrogel films enriched with glucose and yeast extract. The mechanical strength of these hydrogel films and their hydrophilicity (measured as equi-

Table 1 Viability of fungal mycelium segments of different sizes after fragmentation

Fungus	Single hyphae fragments (A)	(A) and loose mycelial structures (B)	Compact mycelial structures (C)
T. versicolor	0.89 ± 0.25	0.76 ± 0.03	1.17 ± 0.27
I. lacteus	1.41 ± 0.37	1.05 ± 0.08	1.19 ± 0.22

Results are presented as viability ratio of three replicates ± s.d. (see Results section).

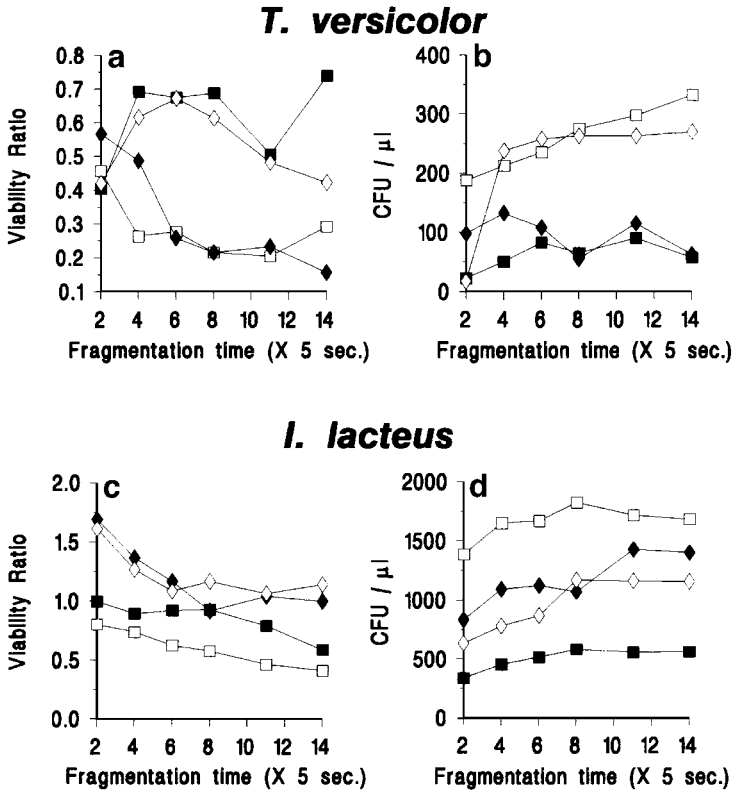

Figure 2 The effect of 3-day (■), 5-day (□), 7-day (◆), and 10-day (◇) old cultures and fragmentation time on the viability ratio (a, c) of mycelial fragments and the number of viable fragments (b, d) of *T. versicolor* and *I. lacteus*. Viability ratio was defined as the ratio between CFU and the number of fragments counted with a haemacytometer. Data represent means of three determinations.

librium moisture content), are shown in Figure 4. Gelatin hydrogel had the highest hydrophilicity, but almost no mechanical strength.

The results after plating on LBA and PDA indicated that the commercial alginate hydrogels had only minor bacterial contamination (Table 2). However, when alginate solutions were incubated at 24°C for 24 h an extensive bacterial contamination developed in some solutions. No contamination with fungi was observed.

The 2% hydrogel films, prepared in triplicate from unsterile commercial alginates supplemented with glucose and yeast extract, were inoculated with 10% (v/v) of 3.939, 2.157, 0.768, 0.1192, and 0.0147 g ml^{-1} of *T. versicolor* mycelial fragments suspension and with 10% (v/v) of 6.542, 3.34, 0.648, 0.3498, and 0.0578 g ml^{-1} of *I. lacteus* mycelial fragments suspension. All films supported the growth of pure fungal cultures without any apparent microbial contamination.

The mechanical strength of alginate hydrogel films varied with the alginate source (Figure 5). Sterilization reduced the strength of some hydrogel films, and increased the strength of others. Concentrations of sterile alginate hydrogels which gave film strengths of approximately 0.5 N were used to determine the viability of mycelial fragments. The viability of *T. versicolor* and *I. lacteus* mycelial fragments in sterile alginate hydrogel solutions depended on the temperature of incubation (Table 3). The highest

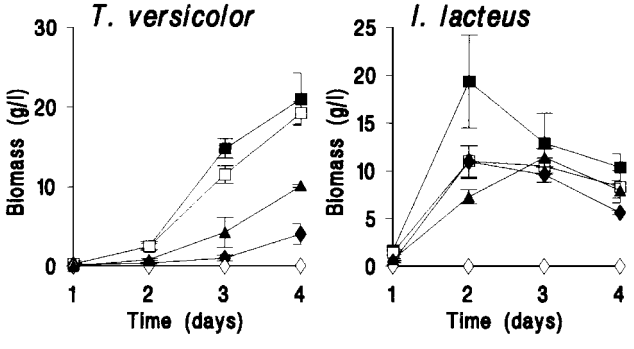

Figure 3 Growth of *T. versicolor* and *I. lacteus* from mycelial fragments on alginate (■), agarose (□), carrageenan (◆), chitosan (◇) and gelatin (▲) hydrogel films. Error bars represent standard deviation of three determinations.

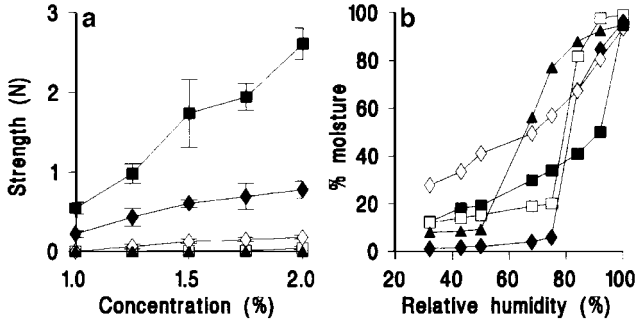

Figure 4 Mechanical strength (a) and hydrophilicity (b), measured as equilibrium moisture content, of alginate (■), agarose (□), carrageenan (◆), chitosan (◇) and gelatin (▲) hydrogel films. Error bars represent standard deviation of three determinations.

Table 2 Bacterial contamination of commercial alginate hydrogels in alginate hydrogel solutions after 24 and 48 h of incubation at 6 or 24°C

Alginate source	(CFU g^{-1}) on					
	LBA 0 h	PDA 0 h	PDA 24 h 6°C	PDA 24 h 24°C	PDA 48 h 6°C	PDA 48 h 24°C
Wego	0	0	0	1.25×10^5	1.25×10^5	0
Danisco	0	1.0×10^3	1.25×10^5	0	1.25×10^5	0
Meer LV	2.0×10^3	0	0	0	2.5×10^5	$>2.5 \times 10^8$
Meer HV	0	0	0	0	0	$>2.5 \times 10^8$
Kelgin XL	0	0	0	8.8×10^7	2.5×10^5	$>2.5 \times 10^8$
Keltex	0	0	0	5.0×10^5	2.5×10^5	$>2.5 \times 10^8$
Kelgin MV	2.1×10^4	1.0×10^3	0	5.0×10^5	0	$>2.5 \times 10^8$
Sigma	0	0	0	0	0	$>2.5 \times 10^8$

Results are expressed as means of three replicates.

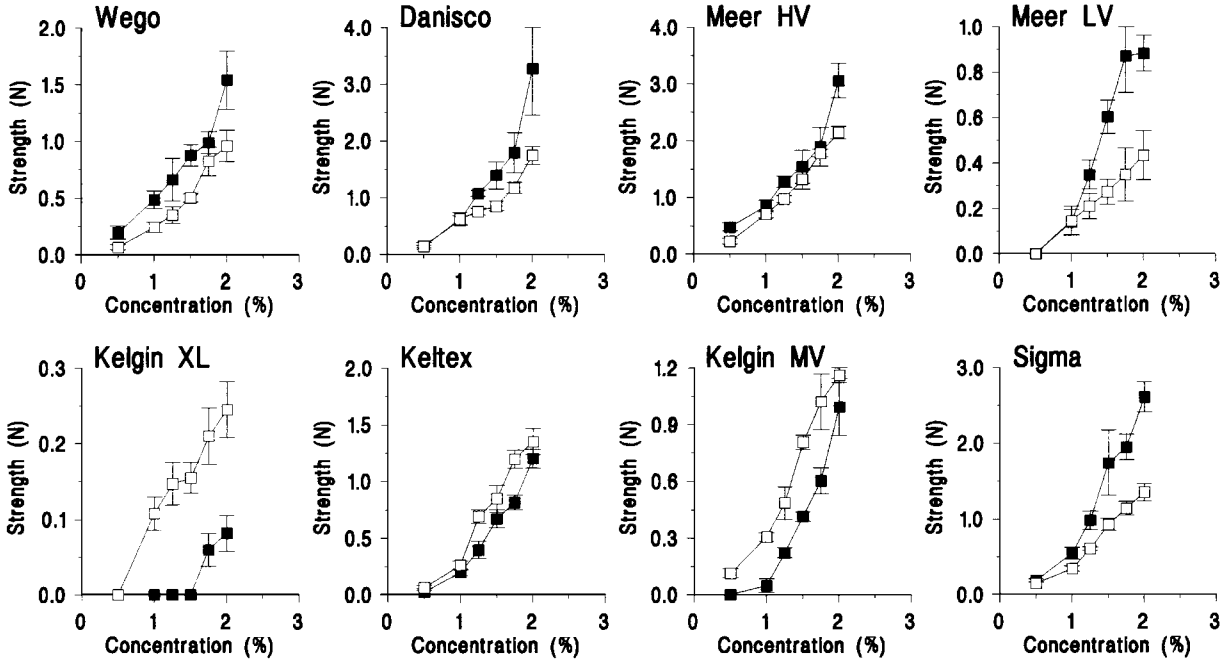

Figure 5 Mechanical strength of 0.5 mm thick alginate hydrogel films determined as force needed to rupture the films. Sterile (■) and non-sterile (□) hydrogel. Error bars represent standard deviation of three determinations.

Table 3 Viability of mycelial fragments incubated for 2 days in sterile 2% solutions of selected commercial alginates, pH 6.5, incubated at 6°C or 24°C

	T. versicolor fragments at		*I. lacteus* fragments at	
	6°C	24°C	6°C	24°C
Wego	7.8 ± 8.9	0 ± 0.0	1.4 ± 2.4	0 ± 0.0
Danisco	44.9 ± 12.8	40.8 ± 7.1	137.8 ± 17.7	56.8 ± 2.7
Meer HV	68.8 ± 14.6	26.0 ± 27.1	77.2 ± 13.8	69.1 ± 2.2
Meer LV	26.3 ± 25.9	15.8 ± 0.0	79.1 ± 6.6	37.7 ± 1.8
Kelgin XL	32.8 ± 13.8	17.1 ± 11.4	83.2 ± 3.1	55.6 ± 2.2
Keltex	18.0 ± 2.8	57.1 ± 6.0	97.3 ± 10.0	66.2 ± 4.0
Kelgin MV	95.8 ± 19.6	121.6 ± 19.6	101.9 ± 2.7	77.9 ± 4.0
Sigma	31.6 ± 7.9	25.3 ± 5.8	73.4 ± 2.6	34.8 ± 2.1

Results are expressed as % of initial CFU (means of three replicates ± s.d.).

mycelial fragment viabilities were determined to be in Danisco, Meer HV, Kelgin MV and in laboratory grade Sigma alginate solutions. Danisco, Meer HV and Sigma alginate hydrogels also had the highest mechanical strengths. Very low fragment viability was observed in the Wego alginate solution. Suspensions with *I. lacteus* mycelial fragments contained more viable units than suspensions with *T. versicolor* fragments. The viability of mycelial fragments in Kelgin MV hydrogel after incubation was higher than that observed initially. This was most likely due to further fragmentation of mycelial fragments (Table 3).

Figure 6 presents the hydrophilicity of sterile Danisco and Meer HV hydrogels, measured as equilibrium moisture content at different RH. Sigma hydrogel was used as a control.

The concentration of Danisco alginate did not influence the growth of *T. versicolor* and *I. lacteus* fragments in hydrogel films (Figure 7b). Growth of *I. lacteus* in alginate hydrogel was inhibited by low pH (Figure 7a) and was higher when the alginate solution was sterilized before gelatinization (Figure 7c). No significant effect of hydrogel pH or sterility on the growth of *T. versicolor* fragments was observed (Figure 7a and c).

Discussion

Viability of microbial propagules is essential for providing the high population density, important for survival of inoculated microorganisms in a target environment [2]. The viability of mycelial fragments did not depend on the size of the fragments (Table 1). Small, single-hyphae fungal fragments (Figure 1a) were viable as were larger pieces of fragmented mycelium (Figure 1b). The viability of mycelium fragments of both *T. versicolor* and *I. lacteus* was affected by culture age and conditions of fragmentation (Figure 2), indicating that these parameters needed to be optimized for each particular fungus.

The suitability of various hydrogels as a medium for fungal propagules and as a microbial carrier was evaluated by measuring the growth and viability of fungal propagules on hydrogel, by testing the mechanical strength of hydrogel films, and by determining the hydrophilicity of the hydrogel. Mechanical strength of hydrogel film would secure complete and equal coating of a pelleted substrate. The strength of the gel-matrix is also an important factor when hydrogel granules are used as a carrier of encapsulated microorganisms for environmental applications [20]. The hydrophilic nature of hydrogels facilitates the growth of microorganisms in environments where relative humidity or moisture content is reduced [7].

The mechanical strength of all hydrogel films except alginate and carrageenan films was very low (Figure 4a). The hydrophilicity of alginate hydrogel was lower than that of carrageenan and of other hydrogels (Figure 4b). However, *T. versicolor* and *I. lacteus* grew better on alginate than on carrageenan or other hydrogels (Figure 3). Earlier,

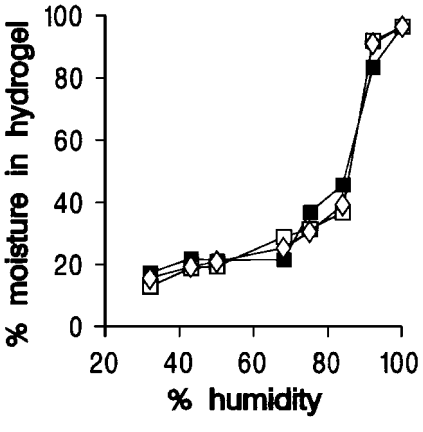

Figure 6 Equilibrium moisture contents for 1% alginate hydrogels at different air relative humidities. Danisco (■), Meer HV (□), and Keltex (◇) hydrogel.

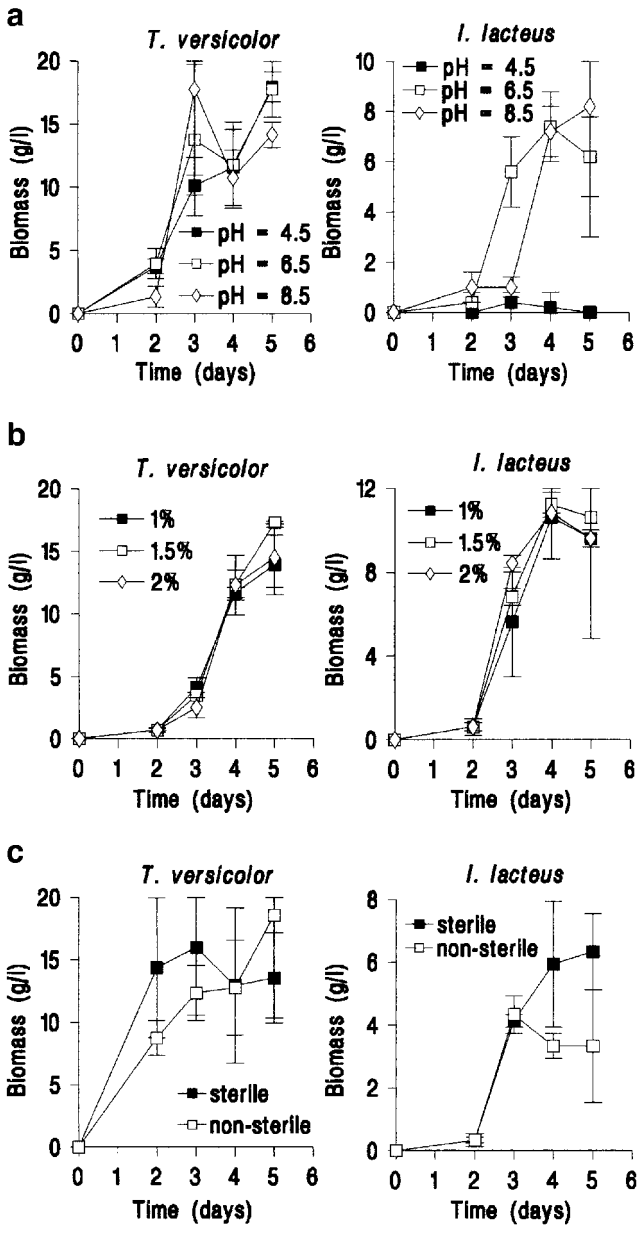

Figure 7 Influence of different pH (a), percent of hydrogel (b), and sterility (c) of Danisco hydrogel on growth of *T. vesicolor* and *I. lacteus* mycelial fragments, expressed as biomass dry weight. Error bars represent standard deviation of three determinations.

it was reported that fungal growth on agar and carrageenan was generally the same [18].

Alginate is a collective term for a family of copolymers containing mannuronic and guluronic acid residues in varying proportions and sequential arrangements. Properties of alginate gels such as strength are determined by such factors as their concentration and monomeric composition [12,22]. Sterilization sometimes resulted in reduction of mechanical strength of gel matrices, probably due to the cleavage of hydrogel monomer units [12] (Figure 5) and would be costly to the operation. All commercial alginates tested had low bacterial and no fungal contamination (Table 2) and supported aseptic growth of entrapped fungal fragments even in unsterilized hydrogels. Sterilization however, had some positive effect on growth of mycelial fragments of *I. lacteus* (Figure 7c). The viability of mycelial fragments incubated in alginate hydrogel solutions, and mechanical strengths of alginate hydrogel films varied significantly for different alginates (Table 3, Figure 5).

The results of this study show that fungal mycelium fragmentation is a suitable way to prepare propagules of some white-rot fungi. Commercially available alginate hydrogels, such as Danisco, which is isolated from the seaweed *Laminaria agitata*, and Meer HV, which is extracted from various species of brown seaweed (genus *Phaeophyceae*), had good mechanical strength (Figure 5) and were able to preserve the viability of mycelial fragments (Table 3). When the properties of these technical grade hydrogels such as pH and sterility are optimized for a particular fungus (Figure 7), and when high hydrophilicity is not essential, they are strong candidates for a medium for applying fungal propagules.

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