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Convenient and effective methods for *in vitro* cultivation of mycelium and fruiting bodies of *Lentinus edodes*

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Three strains were selected from 18 commercial strains of the shiitake mushroom, *Lentinus edodes*, on the basis of rate and density of mycelial growth. Comparison of biomass production between stationary and shake flask cultures was strain-dependent, strain Le-465 producing higher yields from shaken than stationary cultures while no differences were recorded in Le-11 and Le-103. Among 5 media tested for mycelial growth yield, a molasses medium gave the highest biomass after 14 d, but by day 28 yield from that medium was the second lowest. By day 28, a chemically defined medium was the best for biomass yield. Homogenization of mycelia provided an excellent uniform source of inoculum for replicate trials. Homogenization of the inoculum increased the yield of mycelium by day 28, when fruiting started. Chemically-defined media were best for fruiting of *L. edodes*. There was not much difference in fruiting performance between cultures incubated at a uniform 25 °C and those incubated in a 14/22° cycle. Supplementation of flask cultures with vermiculite promoted growth and fruiting. In flask cultures kept in a normal incubator a cotton stopper, as opposed to one made of plastic foam, was most suitable for growth and fruiting. Inclusion of a carbon dioxide trap was not necessary. The best fruiting *in vitro* was obtained by inoculation on to solid supports, like a wire mesh or agar, in crystallizing dishes which were kept within a plastic chamber whose air-flow and humidity could be regulated with air-vents and an air-pump. Under such conditions, vermiculite addition to the media did not enhance fruiting.

Among the various controlled processes for the exploitation of agricultural and forest wastes in the world, none rivals that of the cultivation of edible fungi (Wood, 1985). Global production value has been estimated to be in excess of £2 billion per annum compared with a value of £300 million for enzymes and £5–10 million for microbial pesticides (Wood, 1989).

The black forest or shiitake mushroom, *Lentinus edodes* (Berk.) Sing. [=*Lentinula edodes* (Berk.) Pegler] has become increasingly popular in recent years, cultivation having spread from the Far East to the U.S.A., Canada, New Zealand, Australia and Belgium, with Japan accounting for nearly 70% of the world total in 1985 (Chang & Tan, 1989). The world production of 235 000 tonnes (fresh weight) in 1984 placed it as the most important mushroom after the button mushroom, *Agaricus bisporus* (Lange) Imbach.

Shiitake has traditionally been cultivated on hardwood logs, mainly oak. However, due to the dwindling supply of hardwoods and the long spawn-run period of up to a year before the first harvest, an alternative technique of cultivation on artificial logs consisting of supplemented sawdust, wood chips or other lignocellulosic wastes was developed. This technique was first reported in the late seventies (Fuzisawa, Maedai & Hattori, 1978; Mee, 1978) and has increased in importance. It can provide yields (conventionally expressed in terms of total weight of fresh mushrooms harvested per unit D.W. of substrate) as high as 145% in 6 months (Royse, 1985) compared with 9–35% in 6 years from cultivation on natural logs (San Antonio, 1981; Leatham, 1982).

With the trend towards artificial log cultivation of shiitake, much laboratory research has been directed towards establishing conditions for good growth and fruiting in synthetic culture (Terashita, Kono & Murao, 1980; Kawamura, Goto & Nakamura, 1983; Leatham, 1983; Matsumoto, 1988; Tan & Chang, 1989*a*). A number of different media have been claimed to be optimal for fruiting and some of this research implies that *L. edodes* is extremely fastidious; for example, Leatham (1983) reported that the size of the culture vessel and the medium volume were critical to successful fruiting on a chemically-defined medium. Recent studies have also been directed towards the production of liquid inoculum (Leatham & Griffin, 1984; Song, Cho & Nair, 1987; Raaska, 1989) as a complement to the traditional solid inoculum (Lambert, 1959; Stoller, 1962; Fritsche, 1978; Ito, 1978) as inoculum for mushroom production.

The aims of our present studies are to assess critically the varied approaches published recently to establish convenient and reliable cultivation conditions for mycelia of the shiitake as a source of inoculum (which could also prove useful for commercial inoculum production), and to establish conditions conducive for fruiting *in vitro* to facilitate future biochemical studies.

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MATERIALS AND METHODS

Strain selection

An initial selection was made from 18 commercial (dikaryotic) strains of *Lentinus edodes* (Table 1) on the basis of mycelial density on potato dextrose agar (PDA) slants. Five strains were chosen (Le-11, 31, 46, 103, and 465) and further screened for their vegetative growth rate on PDA agar plates, colony radius being measured with the aid of a colony viewer with a magnified grid (Model LC 30, Chiltern Scientific, England). Cultures were stored in 10% (w/v) glycerol at −70 °C and on PDA slants at 4°.

Medium selection

Six treatments involving five types of liquid media were tested for fruiting *in vitro* using strain Le-11. Two of the media contained the same ingredients (Leatham, 1983) but one was filter-sterilized (as recommended by the original author) and the other autoclaved. The other media were: molasses (3% w/v), new synthetic medium and the yeast extract/malt extract/glucose/peptone mixture (all described by Song *et al.*, 1987), and potato dextrose broth (Ando, 1974).

For standardized tests, 25 ml of media were dispensed into 250 ml conical flasks, with four replicates per treatment. Inoculum was prepared as follows. Two mycelial mats harvested after 21 d growth at 25° on 100 ml of Leatham medium were washed with distilled water, and then homogenized in 150 ml of distilled water for 20 s in a homogenizer (Atomix, MSE, England) at approximately 6000 rpm. One ml of homogenate (equivalent to 3·37 mg dry weight of biomass) was then added to each flask. Flasks were incubated at 25° in an illuminated incubator (Vindon Scientfic Ltd, Oldham, Lancs.) which was set on a 9 h (200–400 lx) light and 15 h dark cycle. Dry weight measurements of the biomass were taken after 14 and 28 d stationary growth.

Table 1. *Lentinus edodes* strains, origins and preliminary qualitative and quantitative screening

Stock number	Source	Growth[a] (quality)	Growth[b] (extent)
Le-2	Dr S. K. Dong, Korea	+ + +	N.D.
Le-11	Tottori Mycological Inst., Japan	+ + + +	61·8 ± 1.6 [B]
Le-13	Dr Tomika, TMI, Japan	+ + +	N.D.
Le-20	Dr J. L. Pang, Taiwan	+ +	N.D.
Le-21	Dr Nair, Australia	+ + +	N.D.
Le-23	Mr S. Nuttalaya, Thailand	+	N.D.
Le-31	Dr T. Yokoyama, Japan	+ + +	45·5 ± 1.1 [C]
Le-37	Professor K. Nakazawa, Japan	+ + +	N.D.
Le-38	Professor S. T. Chang, Hong Kong	+ +	N.D.
Le-42	ATCC 48085	+ + +	N.D.
Le-46	ATCC 48855	+ + +	43·8 ± 1.4 [C]
Le-47	ATCC 48856	+ + +	N.D.
Le-100	Dr K. K. Tan, Singapore	+ +	N.D.
Le-101	Commercial strain, Taiwan	+ + +	N.D.
Le-102	Stock 861120, Taiwan	+ + +	N.D.
Le-103	Mr Inagaki, Japan	+ + +	69·0 ± 1·3 [A]
Le-465	Commercial strain, Taiwan	+ + +	69·5 ± 2.9 [A]
Le-500	Dr K. Y. Cho, Australia	+ +	N.D.

[a] The quality of growth description is based on the apparent visual density of the mycelium, ranging from poor (+) to very dense (+ + + +).

[b] The growth extent is shown as the diameter (in mm) of colonies after 8 d incubation on PDA medium at 25°. Entries show mean ± S.D. of six replicate cultures; means followed by the same letter in square brackets are not significantly different at $P = 0·05$ (Duncan's Multiple Range Test). N.D. = not done.

Stationary versus shake culture

Comparisons of mycelial biomass grown in stationary and shaken cultures were made with the three strains Le-11, 103, and 465. The culture medium was the chemically defined one used by Leatham (1983). Inoculum was prepared as described in the previous section and added to each of 3 replicates of 50 ml medium contained in 500 ml conical flasks. One set of flasks was left stationary in the dark at 25°, while the other set was placed on a gyratory shaker (Model G10, New Brunswick, USA) and shaken at 200 rpm in the same growth room. Biomass was harvested after 21 d incubation.

Effect of homogenization on inoculum performance

Using strain Le-11 and the (autoclaved) Leatham medium (LM), the effect of homogenization was determined. The standard inoculum was prepared as follows. 150 circular plugs of mycelium (7·5 mm diam.) were cut from mycelial mats grown in stationary culture in LM for 21 d and placed into a homogenizer (Atomix, MSE, England) with 300 ml of distilled water. This was equivalent to a tissue concentration of 0·91 mg dry weight ml^{-1}.

The mycelial pieces were homogenized at half-speed (approx. 6000 rpm) sequentially for 30, 60, 90, 120, and 150 s. After each homogenization, 50 ml of the homogenate was transferred to 100 ml conical flasks containing stir bars so it could be mixed during dispensing of 2 ml quantities into each of 4 replicate Petri dishes containing 25 ml of liquid LM. Petri dishes were incubated in the dark at 25°. For a non-homogenized control, mycelial plugs were placed directly into similar dishes. Dry weight determinations were made of the biomass harvested from the various treatments after 7, 14, 21, and 28 d.

When no adverse effects were found following 150 s homogenization, the experiment was repeated with a further comparison between homogenization times of 150 and 600 s. In this experiment the homogenization was conducted at 5° in a cold room with the homogenizer packed in ice.

Viable and total counts were made of the homogenized hyphal suspensions. Viable counts were made by plating dilutions onto PDA plates which were incubated at 25° in the dark. Visible colonies arising from the mycelial fragments were counted after 4 d. Total fragment counts were made using an haemocytometer; a total of 20 independent counts were done on each suspension.

Mycelial fragments from the different homogenization treatments were measured using a videomicrographic system ('Measuremouse', Analytical Measuring Systems, Pampisford, Cambridge) and an Amstrad PC 1520 computer. Ten to twenty fragments from each homogenization treatment were

measured, and the hyphal growth unit, G (Caldwell & Trinci, 1973; Trinci, 1973, 1974; Bull & Trinci, 1977), was calculated for each fragment based on the ratio between the total hyphal length and the number of tips. The mean G values between the different treatments were then compared.

Fruiting of L. edodes *strain Le-11 in different media*

Molasses, yeast extract/malt extract/glucose/peptone (YM-GP), potato dextrose broth (PDP), new synthetic medium (NSM), filter-sterilized Leatham medium (FLM), and autoclave-sterilized Leatham medium (ALM) were dispensed in 25 ml amounts into 250 ml conical flasks. Each flask was inoculated with 1 ml of homogenate prepared by a 20 s homogenization of mycelium. Flasks were incubated at 25° in an illuminated incubator on a 9 h light (200–400 lx) and 15 h dark cycle. After 28 d incubation, one set of 3 replicate flasks per treatment was transferred to another incubator with the same illumination but with a fluctuating temperature regime of 12 h at 14° and 12 h at 22°. Vegetative growth was examined after 30 and 60 d incubation, fruiting was scored up to 90 d. Extent of aerial mycelium and pigmentation were scored qualitatively and the total number of fruiting structures (fs), the time of initiation of fs, and the number of distinctive fruit bodies (fb) produced were recorded. Fs include primordia, buttons, developing or mature (both normal and abnormal) fb, and undefined masses. Distinctive fb were those with a distinct demarcation of pileus and stipe.

Effects of vermiculite and other additives

Homogenates of strains Le-11, 31, 103, and 465 were inoculated into 25 ml volumes of ALM in 250 ml conical flasks, half of which were supplemented with vermiculite (2 g per flask); the flasks were incubated in the regimes described in the previous section. The vermiculite was washed twice with distilled water and oven dried before use. It was obtained from Potters Insulations Ltd, Tameside Mills, Park Row, Duckinfield, Cheshire SK16 5LS, and described as grade L485D.

Activated charcoal (10–18 mesh, activated for gas absorption, from BDH, 10% w/v), glass beads (very fine, approximately 40 mesh from BDH, 40% w/v), and agar (Taiyo powdered agar, from Davis Gelatine, Leamington Spa, England, 3% w/v) were also tested for their effect on growth and fruiting of strain Le-11 cultured on autoclaved LM for comparison with the vermiculite treatments.

Nature of the cultures vessel

The type of culture vessel suitable for fruiting was examined by using crystallizing dishes fitted with different size covers to vary the head space. Six dishes were placed inside a Stewart Propagator Set (Stewart Plastics plc., Surrey, England). This is a domestic device intended for rooting cuttings and preparation of seedlings; it consists of a polystyrene tray with a matching transparent top with two ventilation vents. Additional holes were bored on this top to accommodate two rubber bungs which were connected by plastic tubing to an air pump. Water added to capillary matting in the tray together with adjustment of the air flow enabled r.h. to be varied. Humidity and temperature were continuously monitored with a sensor connected to a digital recorder. The propagator sets were placed into an illuminated cabinet at an ambient temperature between 25 and 30° with a daily 9 h of light (intensity 250–400 lx) for 32 d and then transferred to a constant temperature room at 20° with the same illumination regime.

Statistical analysis

Where the underlying distribution of the data was normal and the experimental variable continuous, a preliminary F test (David, 1952) was used to establish the homogeneity of variances when there were two treatments, and Bartlett's test (Bliss, 1967) was performed if more than two treatments were involved. Once the homogeneity of variances was established a t test or a one-way ANOVA was used to determine the significance of difference between the means if only two groups were involved, or a one-way ANOVA done if more than two treatment means were present (Wardlaw, 1985). If significant differences were found among treatment means, a Duncan's Multiple Range Test (DMRT) (Snedecor & Cochran, 1967) was next employed to extract which of the differences among the means were significant. If the underlying population was discontinuous and non-normal, such as whole number observations involving counts, a χ^2 test was first performed to determine reproducibility between counts (Wardlaw, 1985); this was then followed by a square-root transformation of the count data to normalize their (otherwise Poisson) distribution, after which the transformed values were analysed as a normal distribution (Ashby & Rhodes-Roberts, 1976). Where the data could not be normalized, such as ranks, the data were analysed non-parametrically (Jones, 1973) by comparing medians instead of means using the Friedman test (Campbell, 1974).

Chemicals, reagents and media

All chemicals were of Analar grade and unless otherwise stated were obtained from BDH (Poole, Dorset) or Sigma Chemical Company Limited (Poole, Dorset). Components used in media were from BDH except pure cane molasses which was purchased from Pure Honey Supplies Company, Middlesex, and yeast extract which was obtained from Oxoid Limited (Basingstoke, Hampshire). All solutions were made up with glass-distilled water.

RESULTS AND DISCUSSION

Strain selection

The sources and growth characteristics of the 18 commercial strains are presented in Table 1. The 5 strains selected for further testing were Le-11, 31, 46, 103, and 465. Le-465 and Le-103 had the fastest radial growth rates, but Le-11 was ranked highest for visually-assessed mycelial density, followed by Le-465, Le-46, Le-103, and Le-31 with highly significant differences between their median rank scores. Based on the

Table 2. Biomass (mg D.W.) of *Lentinus edodes* strains obtained from stationary and shaken cultures after 21 d growth at 25° in 50 ml of autoclaved Leatham medium

Strain number	Stationary culture	Shaken culture
Le-11	136·9 ± 14·2	96·0 ± 11·6 [A]
Le-103	25·3 ± 3·3	29·6 ± 1·6 [A]
Le-465	87·5 ± 8·1	262·0 ± 19·9 [B]

Entries show the mean ± S.D. of three replicate cultures.
[A] Data for shaken and stationary culture not significantly different.
[B] Data for shaken and stationary culture significantly different at $P = 0·01$.

Table 3. Biomass yield (mg D.W.) of *Lentinus edodes* strain Le-11 grown in stationary culture in a range of different liquid medium formulations

	Harvested after	
Medium	14 d	28 d
Molasses	37·9 ± 1.7 [A]	49·8 ± 3·1 [B]
New synthetic medium	13·2 ± 0·6 [E]	46·2 ± 3·8 [B]
Leatham medium, filter-sterilized	30·2 ± 2·3 [B, C]	163·7 ± 3·7 [A]
Leatham medium, autoclaved	28·0 ± 1·8 [C, D]	157·2 ± 11·7 [A]
Yeast/malt extracts, glucose and peptone	34·4 ± 0·7 [A, B]	58·7 ± 2·7 [B]
Potato dextrose broth	23·4 ± 0·9 [D]	54·4 ± 8·5 [B]

Entries show mean ± S.D. of four replicates; means followed by the same letter (in a column) are not significantly different at $P = 0·05$ (Duncan's Multiple Range Test). References to the different media are given in the text.

two selection criteria of growth rate and mycelial density, strains Le-465, Le-103, and Le-11 were selected for further studies.

High yields of the mushroom fruit bodies of *Lentinus edodes* have been associated with good mycelial growth in both sawdust (Tan & Chang, 1989*b*) and bedlog cultures (Tokimoto, Fukuda & Tsuboi, 1984) which is why rapid, dense mycelial growth in Petri dish culture was the criterion used for the initial selection of strains. Of the three strains which were selected for further studies, Le-11 was a commercial strain obtained from the Tottori Mycological Institute (Japan) and Le-103 and Le-465 were commercial strains obtained from leading mushroom growers in Japan and Taiwan.

Stationary versus shake cultures

Growth in stationary culture was filamentous while shake culture produced a pelleted growth form. The mean biomass produced from strain Le-11 (136·9 g) in stationary culture, though numerically higher, was not significantly different from the mean biomass (95·0 g) produced from shake culture (Table 2), nor were biomass yields of strain Le-103 significantly different between the two growth conditions. However, with strain Le-465 there was a highly significant difference between biomass harvested from shake culture (262·0 g) and from stationary culture (87·5 g).

Song *et al.* (1987) reported an increase in dry mycelial weight of *L. edodes* with increased shaking frequency up to 150 rpm. However, among the strains we tested, only Le-465 gave a significantly different biomass yield when shaken and stationary cultures were compared. This indicates that this response is strain-dependent. As it is often more convenient to grow mycelia in stationary culture, especially in disposable plates, there seems to be little point in choosing a strain which requires shake culture. Stationary cultures were employed in producing mycelia for inocula and fruit-body production.

Effect of different media on growth

Six treatments, involving 5 chemically-different media which have all been promoted at some time for growing *L. edodes*, were tested. After 14 d incubation differences in biomass yields between the media were not large although the new synthetic medium (NSM; Song *et al.*, 1987) gave a significantly lower biomass yield than the rest (Table 3). The pattern was very much altered after incubation for a further 14 d. At this stage the two Leatham media FLM (filter sterilized) and ALM (autoclaved) produced by far the highest biomass yields with no significant difference between them (FLM, 163·7 g; ALM, 157·2 g), whilst biomass yields from the rest of the media tested were only about one-third of these amounts (Table 3).

Molasses, a natural medium produced from a by-product from the sugarcane industry, has been reported to produce the highest mycelial biomass among 11 media tested after 20 d growth in shake cultures (Song *et al.*, 1987). Addition of 2% molasses to a basal medium of Czapek's solution has been found to stimulate mycelial growth in *L. edodes* (Han *et al.*, 1981) but addition of sucrose to the Leatham medium did not increase growth yield (Tan & Chang, 1989*b*). Malt extract and glucose-peptone-yeast extract were used by Song *et al.* (1987) to produce relatively good yields of shiitake biomass (1·59 and 2·72 mg ml^{-1} respectively among yields ranging from 0·69 to 5·02 mg ml^{-1} from 11 media); and the same authors devised the new synthetic medium (NSM) and reported that it produced the highest yields among a number of media tested in shake flask cultures and within a much shorter time when grown in an airlift fermenter.

The present studies show that different media might be preferred for different purposes. The molasses-based medium was the best for biomass production up to the 14 d harvesting time. The yield on this medium, 7·6 mg ml^{-1} compares well with the 5·0 mg ml^{-1} after 20 d growth in shake cultures reported by Song *et al.* (1987). Further growth in this medium was the slowest, the medium being ranked second lowest in biomass output after 28 d. However, molasses is easy to use, readily available and inexpensive compared to the other semi-defined or defined media, so for the production of biomass over a short incubation time, say for use to prepare a liquidized primary inoculum for example, it would be the preferred formulation. Nevertheless, it would not be suitable as a medium for fruit-body production as much longer incubation times would be needed.

Only the two chemically-defined media were suitable for fruiting *L. edodes* in the laboratory. There was not much difference in fruiting performance between cultures incubated at a constant 25° and those incubated in a 14/22° cycle. Over more extended incubation times, the best biomass yields were undoubtedly obtained using the modification of the defined medium of Wu & Stahmann which was described by Leatham

Table 4. Effect of homogenization of the inoculum on biomass yield of *Lentinus edodes* strain Le-11 grown in stationary culture in autoclaved Leatham medium at 25°

Homogenization time (s)	Day of harvest				
	7	14	21	28	35
			(a) First experiment (four replicates)		
0	4·4 ± 1·4 [D]	53·8 ± 3·0 [C]	89·6 ± 6·5 [C]	174·0 ± 5·4 [C]	
30	13·3 ± 0·6 [A, B]	89·2 ± 4.6 [A]	133·5 ± 4·1 [B]	235·5 ± 10·0 [A, B]	
60	10·8 ± 0.8 [B, C]	85·3 ± 2·8 [A]	138·5 ± 4·4 [B]	221·0 ± 7·8 [A, B]	
90	15·2 ± 0·4 [A]	80·9 ± 2·3 [A]	140·0 ± 3·1 [B]	196·8 ± 15·2 [B, C]	
120	8·6 ± 0·4 [C]	69·1 ± 2·3 [B]	157·3 ± 3·7 [A]	213·9 ± 14·6 [A, B, C]	
150	8·9 ± 0·3 [C]	71·2 ± 2·0 [B]	161·9 ± 4·9 [A]	242·7 ± 22·1 [A]	
			(b) Second experiment (five replicates)		
150	10·2 ± 0·7 [A]	43·4 ± 1·0 [A]	95·6 ± 2·9 [A]	124·8 ± 1·6 [A]	180·7 ± 7.3 [A]
600	6·8 ± 0·4 [B]	39·8 ± 1·4 [A]	89·8 ± 2·6 [A]	121·9 ± 1·0 [A]	171·0 ± 10·2 [A]

Table 5. Characteristics of fragments produced by homogenization

Homogenization time (s)	Visible fragments	Viable fragments	(%) viability	Value of *G* (μm)
30	$6·9 \times 10^4$ [B]	$6·03 \times 10^4$ [C]	87·3 [A]	77·1 ± 7·9 [A]
60	$7·1 \times 10^4$ [B]	$6·05 \times 10^4$ [C]	85·1 [A]	66·0 ± 5·7 [A]
90	$8·3 \times 10^4$ [A, B]	$6·75 \times 10^4$ [B]	81·3 [B]	58·8 ± 3·5 [A]
120	$9·6 \times 10^4$ [A]	$7·65 \times 10^4$ [A]	79·7 [B]	70·9 ± 10.9 [A]
150	$9·2 \times 10^4$ [A]	$7·62 \times 10^4$ [A]	82·8 [B]	N.D.

N.D. = not done. Visible and viable fragment counts were first subjected to a chi-square test to verify reproducibility and that variations were within acceptable stochastic limits. Counts were then converted from Poisson to normal distribution by square-root transformation and differences tested for significance by ANOVA and DMRT.

'*G*' is the hypothetical growth unit (Trinci, 1973) expressed as total hyphal length divided by the number of tips.

(1983). This was true whether the medium was prepared in the manner recommended by Leatham in which the medium was filter-sterilized, or whether it was autoclaved. Autoclaving did not affect biomass yield and caused little if any caramelization and can therefore be recommended as an easy and convenient sterilization method.

Effect of various homogenization times on growth

The biomass weights obtained by growth of mycelia from inocula which had undergone the various homogenization times (0, 30, 60, 90, 120, 150 s) are presented in Table 4*a*. After 7 d incubation biomass yield was significantly higher from inoculum homogenized for either 30 or 90 s than that from 60, 120 and 150 s treatments. All homogenized inocula gave higher biomass yields than the unhomogenized control.

After 14 d incubation biomass differences between the homogenized samples were small (highest being about one-third greater than lowest), but significantly higher yields were recorded for inocula homogenized for 30, 60, or 90 s than for those homogenized for 120 or 150 s. Again, all homogenized inocula gave higher yields than the unhomogenized control. After 21 d incubation, the 120 and 150 s treatments gave significantly higher biomass yields than the others. Differences were again small, the highest yield being 21% greater than the lowest. The biomass yield from the unhomogenized control was only 67% of that from the lowest-yielding homogenized inoculum.

Results from the 28 d harvest revealed that the highest biomass (242·7 g) was obtained from the 150 s homogenization treatment, and the lowest (174·0 g) from the unhomogenized control; the difference between these being highly significant. However, other comparisons imply that although some of the numerical differences can be shown to be significant in pairwise comparisons, biomass yields across all these treatments were essentially similar.

These experiments showed that 2·5 min homogenization in a conventional 'Waring type' blender had no detrimental effect on the inoculum potential of the hyphal suspension, and indeed probably accelerated the initial growth of the culture. To carry the comparison to an unrealistic level, an experiment was performed to compare yields obtained from inocula homogenized for 150 s and 600 s (Table 4*b*). Only the biomass harvested after 7 d revealed a highly significant difference between the yields of the 150 s (10·2 g) and 600 s treatment (6·8 g). At all further harvests, over a 35 d growth period, difference between the yields were not significant.

The effect of homogenization on the mycelia as reflected in the viable and total counts are shown in Table 5. Fragment counts increased significantly with increase in the homogenization period and so did viable counts, although the % viability (ratio of the viable count to the total fragment count) decreased from the 30 s homogenization treatment (87·3%) to the 60 s (85·1%), 90 s (81·3%), 120 s (79·7%) and 150 s treatments (82·8%).

Values of the hyphal growth unit, *G*, calculated from measurements of the hyphal fragments from the various homogenization treatments were not significantly different between the different treatments (Table 5).

Comminution of fungal hyphae for use in inocula has been undertaken by researchers for a long time but there is still uncertainty over its effects. Savage & van der Brook (1946)

found that 2 min of continuous blending with a Waring blender at high speed (10000 rpm) resulted in fragmentation of *Penicillium notatum* and *P. chrysogenum* hyphae primarily at the septa, leaving terminal cells undamaged. No determination of biomass yield or viability were made as they were interested only in a secondary metabolite. Fluorescein diacetate-active soil fungal biomass was found to decrease with increased homogenization time (Söderström, 1979). An evaluation of mechanical chopping of fungal mycelia as a method of increasing biomass was made by Murase & Kendrick (1986), working with *Chaetomium cellulolyticum*. They hypothesized that since mycelial fragments can generate a hyphal tip at each end (Solomons, 1975), increasing the number of tips by chopping should lead to increased growth. They found that chopping for durations from 60–240 s carried out 6 h and 15 h after inoculation resulted in a decrease in yield compared to the unchopped control although no statistical treatment was presented to show whether differences were significant. Leatham & Griffin (1984) reported that homogenized inocula from stationary cultures of *Lentinus edodes* in synthetic medium was inviable when transferred to oak wood and Raaska (1989) also reported the homogenization process to be destructive in *L. edodes*, viability of the fragments being poor. These various studies all seem to imply that homogenization may be detrimental, but we are not aware of any detailed combined analysis of viability and inoculum potential. Homogenization could have important application in liquid inoculum manufacture so it is imperative to establish the precise effects, deleterious or otherwise, which 'blending' causes.

Our results show a significant increase in the number of mycelial fragments with increase in homogenization time although the value of *G* (the average length of hypha associated with each hyphal tip) was not significantly different between the treatments. Whilst there was a significant decrease in viability from the 30 s and 60 s treatments to the longer treatments, there was no effect on biomass yield. Our experiments show that homogenization, under the conditions studied here, had no adverse effects on yield and that the biomass produced from the homogenized mycelia for all the homogenization times were higher than those produced from non-homogenized mycelia. This was true on all days of sampling. The effect of injury suffered by homogenized mycelia was transient, being manifested only during the early stages of growth. This was illustrated by the experiment comparing 150 s and 600 s homogenization in which reduction in yield with the longer homogenization time was evident on day 7 but not thereafter.

Fruiting trials

Fruiting structures (fs) were only produced on FLM, ALM and NSM (Table 6). The number of fs produced on the 25° cultures did not differ greatly from the 14/22° cycled cultures but fs appeared first in the 25° cultures. The most distinctive feature observed was that, in all the strains, there was a great reduction in the amount of pigmentation in media containing vermiculite. Droplet exudation, reported to be connected with fruiting (Leatham, 1985) was not related to production of

Table 6. Effect of medium constitution and temperature regime on fruiting in *Lentinus edodes* strain Le-11

	Number of fruiting structures observed*	
Medium	25°	14°/22° cycle
Molasses	0	0
New synthetic medium	10	10
Leatham medium, filter-sterilized	8	9
Leatham medium, autoclaved	16	17
Yeast/malt extracts, glucose & peptone	0	1
Potato dextrose broth	0	1

* Fruiting structures (fs) include primordia, buttons, developing or mature (both normal and abnormal) fruit bodies, and undefined masses. Entries show the total number of fruiting structures in three replicate cultures; observations were made for 90 d incubation.

Table 7. Effect of vermiculite supplementation (8% w/v) on fruiting of four strains of *Lentinus edodes* grown on autoclaved Leatham medium

	Number of fruiting structures and fruit bodies	
Strain number	With vermiculite	Without vermiculite
Le-11	8 and 6	1 and 2
Le-31	none	none
Le-103	8 and 8	none
Le-465	17 and 6	none

Entries are the means of 4 replicates.

fruiting structures in these experiments. Two strains (Le-465 and Le-103) failed to produce fruiting structures in the absence of vermiculite and in the strain which did fruit in un-supplemented media, more fruiting structures were produced in media supplemented with vermiculite (Table 7). There also appeared to be an association between the increase in number of fruiting structures produced and the absence or reduction of pigmentation in mycelia growing on media including vermiculite. The best fruiting occurred on the vermiculite medium (15 fruiting structures) and the unsupplemented medium (7 fruiting structures), compared with 3, 2, and 1 fruiting structures produced by charcoal-, agar- and glass bead-supplemented media respectively.

Vermiculite is an expanded mica which consists of hydrated magnesium, aluminium and iron silicates. It was rigorously washed and dried before use, so is unlikely to be providing any additional mineral nutrient, but it might either adsorb an inhibitor or provide physical support. Vermiculite has been used before as a supplement for fruiting in shiitake (Miles & Chang, 1987; Tan & Chang, 1989*a*) and has been incorporated into a hydrogel matrix as an inoculum for *Agaricus bisporus* production (Romaine, Nelson & Davis, 1989).

Miles & Chang (1987) used vermiculite originally as a physical support for fruit bodies. As neither agar nor glass beads could equal the fruit-body yield of vermiculite-supplemented media, this is unlikely to be the basis of the favourable effect of vermiculite. Activated charcoal has been shown to remove volatile inhibitors of primordium formation in *A. bisporus* and has been used to obtain primordia *in vitro* (Eger, 1962; Long & Jacobs, 1969; Angeli-Couvy, 1975). No such effect was evident in *L. edodes* so it is unlikely that

Table 8. Effect of culture vessel on fruiting of *Lentinus edodes* strain Le-11 grown on vermiculite-supplemented autoclaved Leatham medium at 25° in 250 ml conical flasks

Treatment	Number of fruiting structures*	Number of fruit bodies	Number of flasks with fs	Time to appearance of fs (d)
Modified flask closure				
Foam stopper	5	2	2	43
Foam stopper + wick	1	0	1	61
Cotton stopper	20	4	4	48
Cotton stopper + wick	11	3	2	60
Foam stopper + cling-film	12	4	4	56
Foam stopper + wick + cling-film	15	1	4	59
Cotton stopper + cling-film	18	6	4	52
Cotton + wick + cling-film	21	4	4	58
KOH trap	9	2	4	54
Water control for KOH trap	8	4	3	41

* Fruiting structures were as defined in Table 6; fruit bodies were those with a distinct demarcation of pileus and stipe. Entries are the means of 4 replicates.

Cotton stoppers consisted of non-absorbent 'cotton wool' wrapped in muslin cloth. Cling-film when applied was wrapped tightly around the plastic foam or cotton stoppers to provide a complete seal to the neck of the flask. The wick consisted of moist absorbent cotton wool suspended from the neck of the flask by thin nichrome wire; moisture was maintained continuously in the cotton by replenishing water lost every other day with (sterile) drops from a Pasteur pipette.

Table 9. Effect of different vessel and cover combinations on fruiting of *Lentinus edodes* strain Le-11 grown on vermiculite-supplemented autoclaved Leatham medium at 25° in 9 cm crystallizing dishes

Treatment	Number of fruiting structures	Number of fruit bodies	Number of vessels with fs	Time to appearance of fs (d)
Loose glass cover	8	0	3	53
Tight plastic cover	19	10	2	39
Matching glass cover	8	6	3	45
Tight fitting 400 ml plastic beaker standing on crystallizing dish	26	15	3	43
Loose fitting 500 ml plastic beaker over crystallizing dish	8	1	3	42

Entries show the totals of 4 replicates.

Table 10. Effect of vermiculite-supplementation on fruiting of *Lentinus edodes* strain Le-11 grown at 25° on autoclaved Leatham medium in 9 cm crystallizing dishes

Treatment	Number of fruiting structures	Number of fruit bodies	Number of vessels with fs	Time to appearance of fs (d)
Without vermiculite	10	2	4	55
+ wire mesh, no vermiculite	19	12	4	50
+ vermiculite	12	1	4	41
+ vermiculite + wire mesh	18	2	4	46
+ vermiculite + agar	18	10	4	47

Entries show the total of 4 replicates.

adsorption of inhibitors accounts for the effect of vermiculite; its mode of action remains obscure.

Effect of the culture vessel on growth and fruiting of Le-11 on LM

Leatham (1983) emphasized the role of the culture vessel in determining fruiting in his experiments with *L. edodes*. Using 250 ml conical flasks, we have compared cotton-wool and foam-plastic stoppers, 'cling-film' sealing, increased humidity promoted by regularly wetted wicks beneath the bungs, and removal of CO_2 by KOH traps.

Fruiting was generally better in flasks with a cotton-wool stopper. Sealing 'cling-film' helped to retain moisture (as judged from the depth of the medium over prolonged incubation) and resulted in better fruiting. However, fruiting performances were reduced in treatments containing wetted wicks. Incorporation of tubes of KOH in the flasks to trap CO_2 did not improve fruiting (Table 8).

In experiments in which the head space was varied, all treatments recorded very good mycelial growth, and all treatments produced fruiting structures (Table 9). These experiments showed that, contrary to earlier reports (Leatham, 1983), *L. edodes* could be successfully fruited in vessels other than conical flasks.

A result which was at variance with earlier experiments was the better fruiting observed in medium without vermiculite compared to vermiculite-supplemented medium (Table 10). The volume of medium (100 ml) used in this experiment was four times the amount used in the flask experiments, and this might have affected the functioning of the vermiculite, but it seems that vermiculite supplementation resulted in better

fruiting only in flask cultures incubated without humidity and aeration controls. In these trials the procedures which gave the best results were those in which mycelial plug inocula were placed on the solid support offered by wire-mesh or agar.

This research has demonstrated that liquid inoculum can be produced in quantity by homogenization with no adverse effect on inoculum potential. We have identified convenient media and growth conditions which permit good yields of mycelial biomass over both long and short incubation periods. By far the best growth and fruiting were obtained by cultivation in crystallizing dishes which were kept within a plastic chamber whose air-flow and humidity could be regulated with air-vents and an air-pump. Inoculation on to solid supports like a wire mesh or agar in these dishes resulted in better growth and fruiting. In these conditions vermiculite addition to the media did not produce the enhancement of fruiting observed for flask cultures.

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