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Estimation of fungal biomass in a solid substrate by three independent methods

S. E. Matcham, B. R. Jordan, and D. A. Wood

Glasshouse Crops Research Institute, Worthing Road, Littlehampton, West Sussex, BN17 6LP, UK

Summary. Dry matter increase of *Agaricus bisporus* mycelium in liquid culture, was found to be directly proportional to quantities of fungal derived chitin and extracellular laccase for up to 28 days following inoculation. Fungal ergosterol showed a similar relationship to mycelial growth which was sustained for 56 days of culture. In cultures grown on autoclaved rye grain a correlation was found between the three biochemical indices and linear extension of the mycelium. Each method gave a similar biomass estimate for cultures grown on cereal grains.

Introduction

The assay of extracellular laccase as a means of estimating mycelial growth of the edible fungus *Agaricus bisporus* on composted wheat straw was described by Wood (1979). It is one of the few methods that have been used to determine growth of a specific fungus in a microbially and chemically complex substrate. In a subsequent study (Wood 1980a) laccase production by *A. bisporus* mycelium in liquid culture was found to vary by a factor of 4 between simple and complex culture media. The possibility of such variation occurring between solid and liquid media raises doubt over the use of the laccase method as a means of accurately determining unknown quantities of *A. bisporus* mycelium in solid substrates.

Other growth linked fungal enzymes may be similarly affected by substrate composition, e.g., fungal esterases, which form the basis of two other methods for determining fungal biomass in solid substrates (Soderstrom 1977; Swisher and Carroll 1980). In a study of the esterases of *Candida lipolytica, aspergillus niger* and an unidentified yeast like fungus, Lloyd et al. (1971) found that esterase activity could not be detected when the organisms were cultured on a solid (agar) mineral-medium containing glucose as a sole carbon source. Esterases *were* found to be present, however, in similar cultures in which glycerol tributyrin was substituted for glucose.

Chitin assay, one of the most commonly used indirect methods for estimating fungal biomass, has been applied to a wide range of solid substrates including living plant tissue, decaying wood, leaf litter, food products and cereal grains (Matcham et al. 1984). The methods of chitin analysis used have varied in rapidity. All involve an initial depolymerisation step following which the yield of glucosamine is quantitated by a colorimetric reaction. Arima and Uozomi (1967) achieved depolymerisation by hydrolysis in 72% v/v H_2SO_4 for 24 h at 26.5° C, other workers have used acid conditions at higher temperatures for shorter periods (Tsuji et al. 1969; Wu and Stahmann 1975). In the current experiments we have used the method of Ride and Drysdale (1972) in which the chitin is part depolymerised under alkaline conditions and the resulting glucosamine is quantified colorimetrically by the method of Tsuji et al. (1969).

The use of chitin determinations to estimate fungal biomass in solid substrates has been the subject of a number of critical appraisals (Sharma et al. 1977; Seitz et al. 1979; Whipps and Lewis 1980). The principal sources of error are the tendency for the chitin content of mycelium to vary with age and the presence of extraneous hexosamines within the substrate which cause interference with the assay.

Ergosterol, the predominant sterol of most fungi, is not produced in significant quantities by green plants. In extracts of plant materials containing other sterols, fungal ergosterol can be measured specifically on the basis of its characteristic UV absorbance and

Offprint requests to: S. E. Matcham

hence be used as an index of fungal colonisation. Seitz et al. (1977) found a close correlation between ergosterol content in samples of cereal grains and measurements of fungal invasion obtained by a plate count method. In a subsequent study (Seitz et al. 1979) ergosterol and chitin contents were compared at intervals throughout growth of *Alternaria* and *Aspergillus* species cultured axenically on milled rice. The two biochemical indices showed a correlation throughout a 21-day incubation period but ergosterol was found to be a more sensitive measurement of the initial stages of colonisation. In the above studies, ergosterol assays were used only as an arbitary index of fungal *growth*, (i.e., relative rate of increase in biomass) the current experiments investigate the use of ergosterol as an absolute measure of fungal *biomass*.

Axenic mycelial cultures of *A. bisporus* grown on autoclaved rye grain ('spawn') are commonly used in mushroom cultivation as an inoculum for the growth substrate, composted wheat straw. We have obtained three independent estimates of mycelial biomass in such cultures by the determination of fungal chitin, ergosterol, and extracellular laccase. Considerations relevant to the wider application of these methods are discussed.

Materials and methods

Liquid cultures

Liquid cultures were grown on malt extract medium (ME) (Wood 1976). A series of 250-ml Erlenmeyer flasks containing 50 ml of ME medium were each inoculated with 0.5 ml of a suspension of *A. bisporus* hyphal fragments (Fermor and Wood 1981) and incubated statically at 25° C for 56 days. At weekly intervals following inoculation replicate flasks were harvested for determination of mycelial dry weight, laccase activity, chitin, and ergosterol content.

Grain cultures

Rye grain was immersed in water and boiled for 20–30 min, excess water was then drained and discarded. The grain was then mixed with 0.5% w/w powdered chalk and 2% w/w gypsum. Into each of 50 boiling tubes was dispensed 25 g of grain (moisture content 54%) prepared as described above. The tubes were closed with aluminium caps and autoclaved for 2 h. Single 16-mm diameter agar plugs cut from ME agar previously colonised with *A. bisporus* mycelium were used to surface inoculate the grain and the cultures were incubated at 25° C in a closed polythene box 28 × 14 × 21 cm lined with moistened paper towelling. Linear extension of the mycelium was measured in each tube at suitable intervals throughout colonisation and sample cultures were harvested simultaneously for laccase, chitin and ergosterol assay. Triplicate cultures were taken for each assay at each sample data.

Sample preparation and extraction

Liquid cultures harvested for laccase assay were vacuum filtered through pre-weighed 9 cm diameter Whatman GF/A glass microfibre discs which were then dried to constant weight at 100° C for mycelial dry weight determination. The cell free supernatant was retained for laccase assay. Mycelial mats for chitin and ergosterol assay were harvested from the liquid medium by centrifugation for 20 min then vacuum filtered through a grade-1 Pyrex sinter. The grain residue was discarded.

Laccase was extracted from the grain cultures by grinding the total contents of each tube with a mortar and pestle and mixing with 50 ml distilled water. The resulting slurry was steeped for 20 min then vacuum filtered through a grade-1 Pyrex sinter. The grain residue was discarded.

Grain samples for both chitin and ergosterol assay were dried to constant weight at 100° C and milled to a uniform fine powder using a Glenn Creston ball mill. Starch content of samples for chitin assay was reduced by digestion with diastase (mixed α and β amylase) 10 ml of a stock solution containing 40 enzyme units/ml diastase (BDH Chemicals, Poole, England) in 0.1 M phosphate buffer pH 7.0 was mixed with a 0.1-g sample of dried, milled, fungal colonised grain in a 50-ml polypropylene centrifuge tube. The mixture was incubated in a water bath at 25° C for 3 h. After digestion the tubes were centrifuged for 20 min at 12,000 *g* and the supernatant discarded.

Extracts for ergosterol assay were prepared as follows. Total lipids were extracted from liquid cultured mycelium by the method of Garbus et al. (1963). The mycelium was homogenised in a 2 : 1 (v : v) mixture of methanol and chloroform the homogenate was allowed to stand for a minimum of 30 min after which one volume each of H_2O, chloroform and 2 M KCl in 0.5 phosphate buffer, pH 7.4 were added. This established a two-phase aqueous/chloroform system from which the lipid containing chloroform phase was separated and retained for further clean-up. The aqueous phase was discarded.

Samples of dried, milled grain were extracted by mixing with ethanol in the proportion of 2 ml ethanol to 1 g grain, the mixture was steeped for 1 h, then the supernatant was decanted and the residue washed with two further aliquots of ethanol. Following clarification by centrifugation for 10 min at 2,000 *g*, the three washings were combined.

Both sets of extracts were evaporated to dryness under oxygen free nitrogen prior to saponification in a solution of 1 N KOH in 95% v/v ethanol for 1 h at 70° C (Christie 1976). After cooling, the mixtures were diluted with two volumes of water and the non-saponifiable fraction containing ergosterol extracted with three washings of diethyl ether or petroleum ether. The washings were combined and dried over sodium sulphate prior to assay.

Assay procedures

Laccase was assayed by the oxygen electrode method (Wood and Goodenough 1977). Samples (0.1 ml) of the liquid culture supernatant or grain extracts were mixed with 2.6 ml sodium acetate-acetic acid buffer 0.1 M pH 5.6 in the electrode chamber. The reaction was initiated by the addition of 0.3 ml 0.1 M p-phenylenediamine. Components of the reaction mixture were maintained in a water bath at 25° C prior to assay. Activity was calculated from the initial rate of oxygen consumption. The unit of activity was defined as the amount of enzyme which consumed 1 μmol $O_2 \cdot min^{-1}$.

Chitin was assayed by the method of Ride and Drysdale (1972). Samples were hydrolysed in concentrated KOH (120 g KOH dissolved in 100 ml H_2O) by autoclaving for 15 min at 120° C. The resulting chitosan suspension was precipitated and washed with progressive dilutions of ethanol in water then deaminated and solubilized with nitrous acid formed by the addition of $NaNO_2$ and $KHSO_4$. Colorimetric assay followed using 3-methyl-2-benzothiazolone hydrazone hydrochloride (MBTH) and $FeCl_3$ and was standardized with glucosamine HCl stock solutions. The chitin values were calculated as glucosamine HCl equivalents. Chitin data

from colonised grain was adjusted to take account of the hexosamine content of grain prior to inoculation (0.26 mg/g dry wt. grain) which was determined by averaging the chitin assay of six replicate samples.

Extracts of liquid cultured mycelium were assayed for ergosterol by GLC using a Pye Unicam Model. 204 gas chromatogram with a OV17/Chromosorb W column or by U.V spectroscopy. Grain extracts were assayed by UV spectroscopy.

In preliminary studies the non-saponifiable fraction of the grain extracts was separated,prior to UV spectroscopy, by a TLC method similar to that described by Seitz et al. (1977). Plates of silica gel-G (activated 1 h at 100° C) were developed with a solvent system consisting of light petroleum bp 40–60° C : diethyl-ether : acetic acid, 90 : 10 : 1 (v : v : v). Component bands were visualized by spraying with a 0.1% w/v solution of dichlorofluor-escein, the sterol band was scraped from the plate and extracted with ethanol.

Subsequently this additional separation step was abandoned and the extracts were assayed directly after saponification. In these later assays ergosterol was differentiated from background UV absorbing material by means of electronically derived second derivative spectra using a Perkin-Elmer Model 557 spectrophotometer.

Results

In liquid cultures laccase and chitin were found to increase in direct proportion to mycelial dry weight for the initial 28-day growth period (Figs. 1 and 2). The correlation coefficients were: laccase $r = 0.96$; chitin $r = 0.97$. For ergosterol the correlation remained linear up to 56 days following inoculation (Fig. 3) $r = 0.997$.

In grain cultures a similar relationship was found between the three chemical indices and linear extension of the mycelium (Figs. 4–6). Correlation coefficients were: laccase $r = 0.966$; chitin $r = 0.962$; ergosterol $r = 0.933$.

From the liquid culture data the following conversion factors (CF) were obtained: laccase

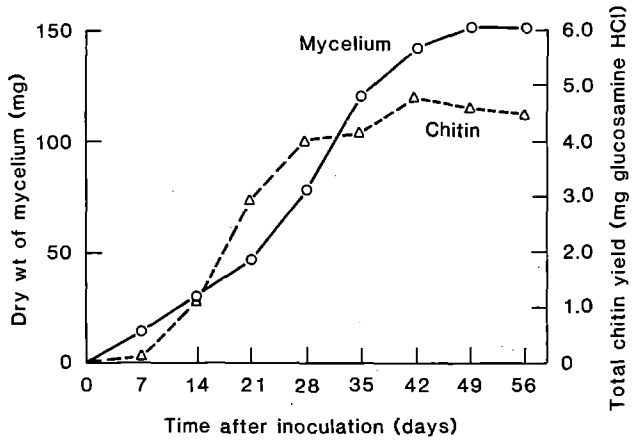

Fig. 2. Mycelial dry wt and chitin yield per 50 ml ME liquid medium in cultures of *Agaricus bisporus;* O———O, mycelial dry wt.; △———△, total chitin

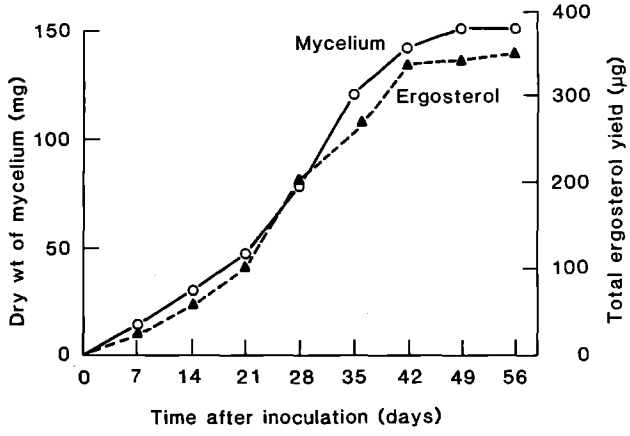

Fig. 3. Mycelial dry wt and Ergosterol yield per 50 ml ME liquid medium in cultures of *Agaricus bisporus;* O———O, mycelial dry wt.; ▲———▲, total ergosterol

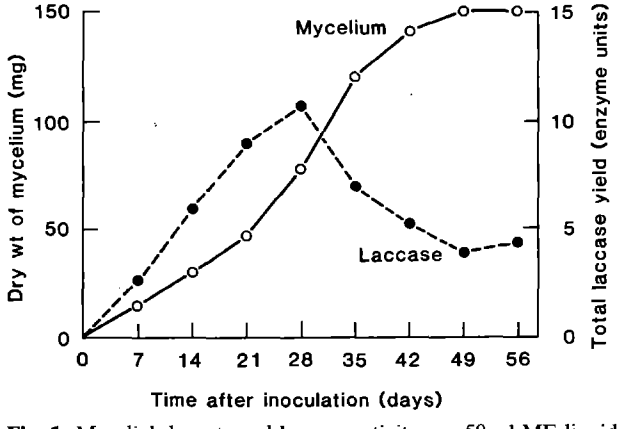

Fig. 1. Mycelial dry wt. and laccase activity per 50 ml ME liquid medium in cultures of *Agaricus bisporus;* O———O, mycelial dry wt.; ●———●, laccase activity

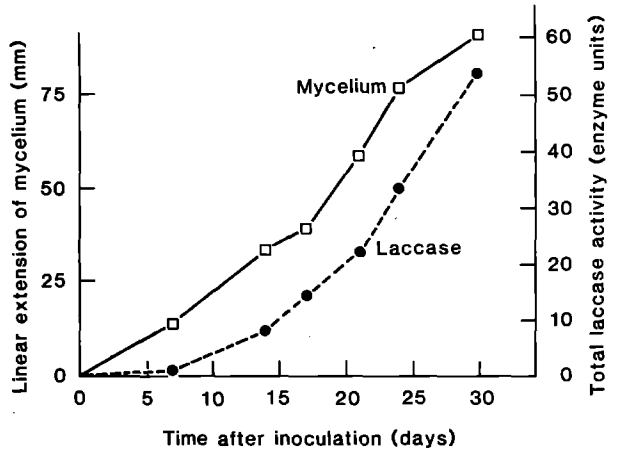

Fig. 4. Linear extension of *Agaricus bisporus* mycelium on autoclaved rye grain and Laccase activity per 25 g fresh wt. grain; □———□, mycelial extension; ●———●, laccase activity

0.178 enzyme units/mg dry wt. mycelium; chitin 0.089 mg/mg dry wt. mycelium; ergosterol 2.2 μg/mg dry wt. mycelium. The CF for chitin was calculated as the mean chitin content of the liquid cultured mycelium during the period of linear correlation between chitin and mycelial dry weight (i.e., the mean of the values from four sampling dates). CFs for ergosterol and laccase were calculated on a similar basis.

Using these CF values we obtained estimates of mycelial biomass for the fully colonised grain tubes at 30 days from inoculation (see Table 1).

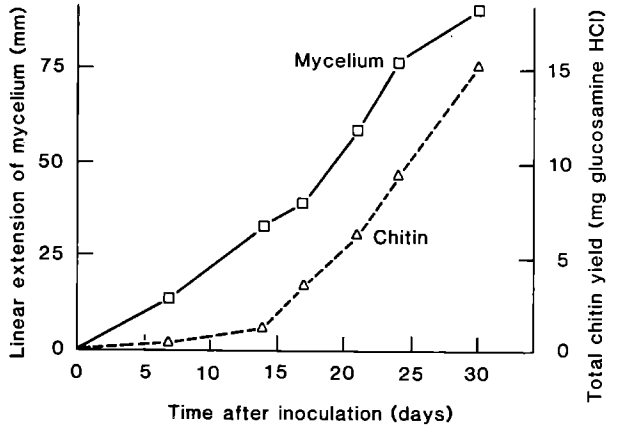

Fig. 5. Linear extension of *Agaricus bisporus* mycelium on autoclaved rye grain and Chitin content per 25 g fresh wt. grain; □——□, mycelial extension; △——△, chitin content

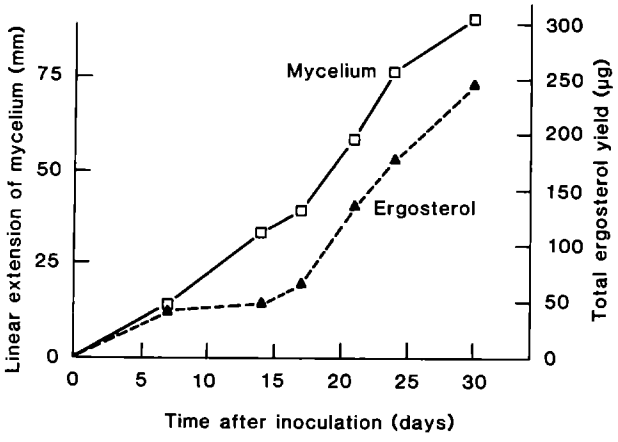

Fig. 6. Linear extension of *Agaricus bisporus* mycelium on autoclaved rye grain and ergosterol content per 25 g fresh wt. grain; □——□, mycelial extension; ▲——▲, ergosterol content

Table 1. Estimated mycelial content of colonised grain by the three assay methods

Mycelial content (mg dry weight mycelium/g dry weight grain)		
Laccase method	Chitin Method	Ergosterol method
26	17	10

Discussion

The three biochemical indices showed a close correlation to mycelial growth and gave similar estimates of fungal growth in colonised grain. There are however a number of factors that limit the wider application of these methods, including the chemical composition of the substrate (i.e., possible presence of interfering substances); the desired degree of sensitivity and in the case of substrates such as soils and composts which contain complex microbial populations; the ability to distinguish the fungus being studied from other micro-organisms present. When the nature of the substrate permits the use of more than one method, as in the current study, procedural considerations such as rapidity of the assay protocol and the availability of required instrumentation may decide the final choice.

The relationship between laccase and growth of *A. bisporus* mycelium in our experiments was similar to that reported by Wood (1979) except for the decline in enzyme activity which occured in liquid cultures after 28 days of incubation. This may have been due to the action of proteases released during senescence of the mycelium or to physiological control as was observed by Wood (1980b) in fruiting cultures of *A. bisporus* during development of basidiocarps. In the grain cultures there was no decline in laccase during the period of the experiment and from the evidence of the two other biochemical indices, the biomass estimate for the colonised grain obtained by the laccase method appears realistic. When liquid cultures are used to standardise enzymic methods for determining fungal biomass in solid substrates the liquid media should ideally be nutritionally analogous to the solid substrates being studied. This may be achieved in some instances by preparing the liquid media from extracts or digests of the solid substrates. In applications where it is not possible to convert growth linked enzyme activities into reliable estimates of biomass, enzyme assays may still provide a useful relative index of fungal growth.

The chitin assay was the most lengthy method assessed, taking in excess of 7 h to assay batches of 16 samples. By comparison the ergosterol method took 5–6 h and could be readily scaled up to cope with larger numbers of samples within the same time scale. Laccase assays could be performed at the rate of 12 samples in 1 h. The only alternative method of chitin analysis offering a significant improvement in rapidity is that which was described by Wu and Stahmann (1975) utilizing an automatic aminoacid analyser. This latter method may have the further advantage of being less prone to chemical interference, although in common with other means of chitin analysis it will

register glucosamine that is not of fungal origin. In an investigation of the growth of *A. bisporus* on composted wheat straw quantitative assessment of mycelial biomass using the chitin method was rendered totally impracticable by high background levels of glucosamine in the compost prior to inoculation with the fungus (Matcham et al. 1984). In cereal grains levels of endogenous hexosamines though relatively low, are sufficient to reduce the sensitivity of the chitin method, i.e., the ability to detect low levels of fungal growth.

Both the methods of ergosterol analysis employed have their own limitations. The GLC method though proving satisfactory for determining the ergosterol content of liquid cultured mycelium, could not be applied to rye grain because sterols endogenous to the grain have similar GC retention times to ergosterol. Direct UV spectroscopy, though distinguishing ergosterol from other sterols, proved unsatisfactory for assaying grain extracts because of a high background of UV absorbance from other substances. Ergosterol's distinctive spectral properties enabled us to quantify it against this background by means of second derivative spectra.

Ergosterol appeared to be a more sensitive indicator of low levels of mycelial growth than either chitin or laccase, showing a substantial initial rise in the grain cultures after 7 days of incubation (Fig. 6). Laccase and chitin though measurable at this stage were closer to the lower limits of detection. The assay of ergosterol would also appear to be less prone to chemical interference than that of chitin and hence may be applicable to a wider range of substrates. The HPLC method favoured by Seitz et al. (1977) which separates the ergosterol from other hydrophobic components and quantitates on the basis of UV absorbance at 282 nm offers a further improvement in specificity. The authors regret that due to the lack of suitable equipment we were unable to utilize this method in the current study.

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