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Quantifying Mold Biomass on Gypsum Board: Comparison of Ergosterol and Beta-*N*-Acetylhexosaminidase as Mold Biomass Parameters

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Two mold species, *Stachybotrys chartarum* and *Aspergillus versicolor*, were inoculated onto agar overlaid with cellophane, allowing determination of a direct measurement of biomass density by weighing. Biomass density, ergosterol content, and beta-*N*-acetylhexosaminidase (3.2.1.52) activity were monitored from inoculation to stationary phase. Regression analysis showed a good linear correlation to biomass density for both ergosterol content and beta-*N*-acetylhexosaminidase activity. The same two mold species were inoculated onto wallpapered gypsum board, from which a direct biomass measurement was not possible. Growth was measured as an increase in ergosterol content and beta-*N*-acetylhexosaminidase activity. A good linear correlation was seen between ergosterol content and beta-*N*-acetylhexosaminidase activity. From the experiments performed on agar medium, conversion factors (CFs) for estimating biomass density from ergosterol content and beta-*N*-acetylhexosaminidase activity were determined. The CFs were used to estimate the biomass density of the molds grown on gypsum board. The biomass densities estimated from ergosterol content and beta-*N*-acetylhexosaminidase activity data gave similar results, showing significantly slower growth and lower stationary-phase biomass density on gypsum board than on agar.

In moist buildings mold growth is suspected to cause health problems. Methods allowing quantification of mold on building materials are therefore important when evaluating mold damage in buildings and the quality of the remediation efficacy. Determination of the ergosterol content is commonly used to estimate fungal biomass in various environments (1, 4, 10, 11) and has been shown to be a suitable marker for estimation of fungal concentrations on building materials contaminated by mold (7). However, determination of the ergosterol content is rarely used in practice for investigations of building materials as it requires advanced equipment and highly trained personnel. Instead, cultivation methods and microscopy of tape lift samples with counts of fungal propagules (conidia, hyphae, conidiophores) are used. Quantification of mold biomass by microscopy may be influenced by a high degree of observer subjectivity (5, 12) and interference due to debris and fibers and can only be performed by trained microbiologists. Cultivation of mold from building materials usually takes 4 to 7 days and is not well suited for quantification of fungal biomass since it has been shown to be mainly a measure of sporulation and since spores only constitute 0 to 5% of the total fungal biomass (10).

Beta-*N*-acetylhexosaminidase activity was shown to correlate with the fungal index molecules ergosterol and the phospholipid fatty acid 18:2ω6 in soil samples (4). A method for detection and quantification of mold biomass on building materials based upon fluorogenic detection of beta-*N*-acetylhexosaminidase activity has been developed (the MycoMeter test) (9). The method is much simpler to perform than ergosterol detection and can be performed on-site.

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This study compares measurements of fungal biomass growing on agar and on gypsum plates, measured as both biomass dry weight and ergosterol and enzyme activity.

MATERIALS AND METHODS

Fungal strains. *Stachybotrys chartarum* (IBT 9695) and *Aspergillus versicolor* (IBT 16000) were obtained from the culture collection of Biocentrum, Technical University of Denmark.

Substrate and inoculation. The inoculum cultures were grown for 6 weeks on Czapek yeast (autolysate) extract agar medium. Suspensions of spores from these cultures were made by adding 10 ml of sterile tap water with 0.01 ml of Tween 80 to an agar plate and stirring with a Drigalski spatula. The spore suspensions were then filtered through a nylon filter (41-μm pore size), and the filtrate was diluted to a final volume of 40 to 80 ml.

A V8 agar medium (2) was used for the experiments on agar plates (9- by 9-cm square). Sterilized cellophane membranes were placed on top of the agar medium (8). The spore solution (0.2 ml) was then added to the agar plates and the spores were distributed evenly by stirring with a Drigalski spatula.

Gypsum board with wallpaper was cut into discs with a diameter of 11.5 cm. These discs were placed in 16-cm-diameter plastic containers, and 45 ml of sterile tap water was added. After 24 h the water had been absorbed by the gypsum discs. A total of 12 discs were inoculated with each mold species. The discs were then inoculated using the same procedure as described for the agar plates except that 0.25 ml of the suspension was used and the use of cellophane membranes was omitted. After inoculation the discs were incubated at 25°C in a glass desiccator (inner diameter, 30 cm) to which 500 ml of sterilized distilled water had been added to maintain humid conditions.

Measurements of growth. A 9-cm^2 sample was cut from the agar plates overlaid with cellophane, and the mold was gently washed off the cellophane onto a preweighed membrane filter and dried at 80°C for 24 h for determination of biomass dry weight (6). A 1-cm^2 sample was cut for determination of enzyme activity and ergosterol content. Three replicates were used for determination of biomass dry weight, enzyme activity, and ergosterol content. On days 0, 1, and 2 the three replicates were pooled for detection of biomass dry weight.

Circular plugs (diameter, 1 cm; thickness, 1 to 2 mm) were sampled from the gypsum plates with a cork bore. The plugs contained the wallpaper, the paper from the gypsum plate, and a small amount of attached gypsum. The plugs were transferred into a 5-ml vial. Enzyme activity and ergosterol content were deter-

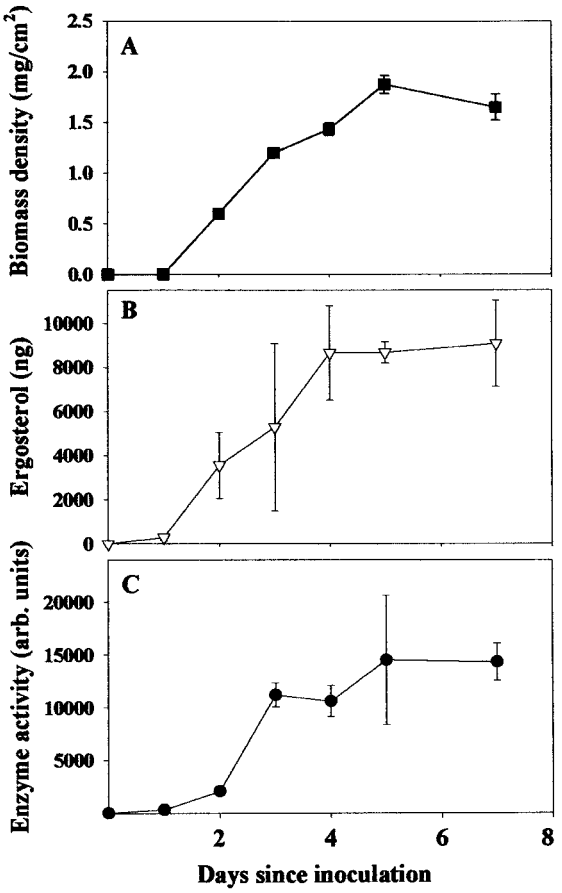

FIG. 1. Biomass density (dry weight) (A), ergosterol content (B), and β-*N*-acetylhexosaminidase activity per cm^2 (C) of *S. chartarum* grown on V8 agar overlaid with a cellophane membrane.

mined from the same plug. At each sampling time three replicates were used for determination of ergosterol content and enzyme activity. The replicates were sampled from three different gypsum discs. The experiments on both agar and gypsum board have been conducted twice, with similar results.

The beta-*N*-acetylhexosaminidase activity was measured, typically within an hour after sampling, by use of a modified version of the MycoMeter protocol (MycoMeter handbook, September 2001). One milliliter (enough to cover the plug) of the fluorogenic enzyme substrate solution was added to each container. After 30 min of incubation at ambient temperature, the enzyme activity was quantified by measuring the fluorescence on a Picofluor fluorometer (Turner Designs, Sunnyvale, Calif.) according to the instructions given in the MycoMeter protocol. After determination of the enzyme activity, the samples were stored at −20°C for ergosterol quantification.

Ergosterol content was determined by a slightly modified version of the method previously described (6). A mixture of 4.0 ml of methanol, 1.0 ml of 2.0 M NaOH, and 100 μl of 1.0-μl/ml [4-$^2H_2$]ergosterol was added to each vial. The samples were hydrolyzed at 85°C for 90 min. After cooling to room temperature, the ergosterol was extracted twice with 0.8 ml of pentane evaporated to dryness, and the sample was derivatized using pyridine–*N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (1:1). Samples were then redissolved in 50 μl of toluene and analyzed using gas chromatography-tandem mass spectrometry (GC-MS/MS) (Finnigan GCQ). Separation was performed on a 0.10-μm diameter, 30-m long HP-5 Trace column (Hewlett-Packard, Avondale, Pa,). *m*/*z* 363 and 365 were used as parent ions and *m*/*z* 157 and 159 were the daughter ions for ergosterol and [4-$^2H_2$]ergosterol, respectively.

RESULTS

S. chartarum was inoculated onto agar covered with cellophane, which allowed for determination of biomass density. Figure 1 shows biomass density, ergosterol content, and beta-*N*-acetylhexosaminidase activity monitored over a 7-day period. Approximately 5 days after inoculation, the biomass density reached a maximum (stationary phase) of 1.7 mg per cm^2. Linear regression analysis of data from days 1 to 7 showed that the biomass density correlated with both ergosterol content ($r^2 = 0.968$; $P < 0.001$) and enzyme activity ($r^2 = 0.935$; $P < 0.001$). Experiments with *A. versicolor* gave similar results with respect to both maximum biomass density (1.4 mg per cm^2) and correlation to ergosterol content ($r^2 = 0.922$; $P < 0.001$) and enzyme activity ($r^2 = 0.968$; $P < 0.01$). The coefficients of variation were 15 to 37% for ergosterol content and 21 to 28% for enzyme activity. Conversion factors (CF) for both ergosterol content and enzyme activity were calculated from the slope of the linear regression (Table 1) and used for estimating the biomass dry weight density of molds on the gypsum board.

With gypsum board both ergosterol content and enzyme activity data showed that the molds grew significantly slower, taking longer to reach maximum biomass density (Fig. 2). The estimated maximum biomass density from determinations of ergosterol content and enzyme activity on the gypsum board yielded values in the same order of magnitude for the two species (Table 1) and was around a quarter to a third (on average 23% for *A. versicolor* and 32% for *S. chartarum*) of that obtained on agar.

The detection limits were determined to be approximately 10 ng for ergosterol detection and 12 arbitrary fluorescence units for enzyme activity. Values above the detection limits were generally seen earlier with the enzymatic assay than with the ergosterol content analysis.

DISCUSSION

The present study has shown a linear correlation between both ergosterol and β-*N*-acetylhexosaminidase activity and the actual biomass density measured by weighing the mold growing on agar plates covered with cellophane. This confirms the previously described correlation between β-*N*-acetylhexosaminidase activity and ergosterol content in soil samples (4) as well as demonstrates that the enzyme is growth related, representing the fungal biomass in both the growth and the stationary phase.

The use of enzyme activity as a fungal biomass indicator has previously been attempted, and a good correlation was found between laccase activity and the biomass of *Agaricus bisporus*

TABLE 1. CFs and estimated biomass densities for growth on gypsum board

Organism	CF[a] for:		Estimated biomass density (mg/cm^2) using:	
	Ergosterol	Enzyme activity	Ergosterol CF	Enzyme activity CF
S. chartarum	5,078	8,275	0.26	0.38
A. versicolor	5,410	12,370	0.52	0.55

[a] CFs for ergosterol are in nanograms of ergosterol per milligram of biomass dry weight; CFs for enzyme activity are in arbitrary fluorescence units formed per milligram of biomass dry weight per 30 min.The CFs were calculated from linear regression analysis of agar data from days 1 to 7. The CFs were used to estimate the stationary-phase biomass densities on the gypsum board.

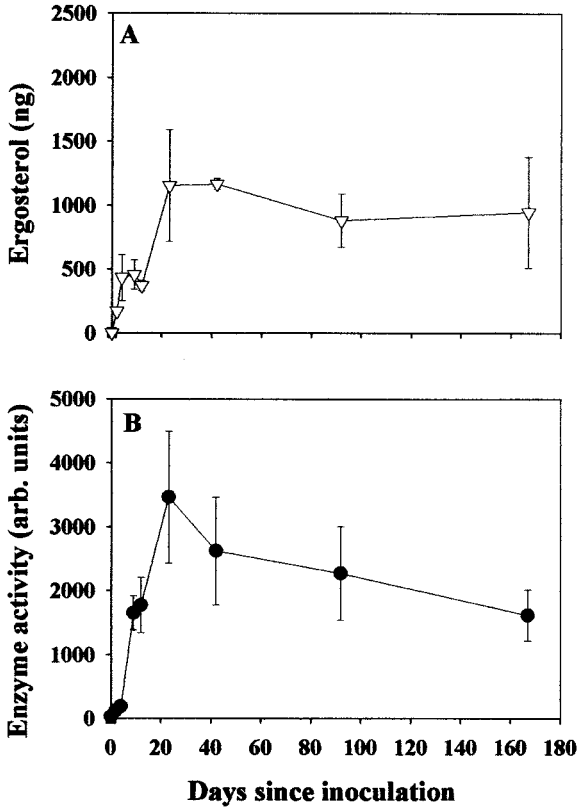

FIG. 2. Ergosterol content (A) and β-*N*-acetylhexosaminidase activity (B) of *S. chartarum* grown on wallpapered gypsum board.

on rye grain (3). However, not all filamentous fungi possess laccase activity, which excludes the use of laccase activity as a general indicator of fungal biomass. Unlike laccase, β-*N*-acetylhexosaminidase activity appears to be present in all filamentous fungi, as it has been found in each of 42 arbitrarily chosen species (unpublished results).

The CF for ergosterol found in the present study (5.1 to 5.4 μg of ergosterol/mg of biomass dry weight) is within the same range as was earlier reported (1, 3, 11). Seitz et al. (11) found an ergosterol content of 2.3 to 5.9 μg/mg of dry weight in three fungi grown in liquid cultures in malt extract medium, and Matcham et al. (3) found 2 to 2.7 μg of ergosterol/mg of dry weight in *Agaricus bisporus* grown in liquid cultures on malt extract medium. The average ergosterol content in 12 aquatic hyphomycetes was 5.5 μg/mg of dry weight (ranging from 2.3 to 11.5 μg/mg of dry weight) (1). The CFs found in the present study were established on agar medium and may not be representative when fungi are growing on different building materials such as gypsum board. Other factors, such as interspecies differences, age of the mold growth, and growth conditions in general, may affect the CFs and therefore limit the accuracy of the biomass density estimates. The data suggest that the interspecies difference may be more significant for the enzyme activity than for the ergosterol content.

While estimation of biomass density from CFs may have limitations when it comes to very accurately predicting the fungal biomass density, the present study has shown that ergosterol content and beta-*N*-acetylhexosaminidase activity measurements yield the same information on the time course of fungal growth and the level of biomass density obtained. Quantification of ergosterol is used in scientific studies of mold on building materials but requires advanced equipment and highly trained personnel, which limits the use of ergosterol measurements in mold remediation cases. The beta-*N*-acetylhexosaminidase activity method can be performed on-site with relatively simple equipment and is therefore a useful and convenient alternative for quantification of mold biomass on building materials.

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