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Comparison of Methods for Estimating the Biomass of Three Food-Borne Fungi with Different Growth Patterns

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To evaluate the effectiveness of steps taken to reduce the growth of molds in food and feed, methods that can accurately quantify the degree of fungal contamination of solid substrates are needed. In this study, the ergosterol assay has been evaluated by comparing the results of this assay with spore counts and hyphal length measurements made with a microscope and with CFU counts. Three fungi with different growth patterns during cultivation on a synthetic agar substrate were used in these experiments. For the nonsporulating *Fusarium culmorum*, there was good agreement between changes in hyphal length, CFU, and ergosterol content. *Penicillium rugulosum* and *Rhizopus stolonifer* produced many spores, and the production of spores coincided with large increases in CFU but not with increases in hyphal length or ergosterol content. Spores constituted between 3 and 5% of the total fungal mass. Changes in ergosterol level were closely related to changes in hyphal length. It was concluded that ergosterol level is a suitable marker for use in quantitatively monitoring fungal growth in solid substrates.

Fungi cause serious problems during food and feed storage by producing mycotoxins and potentially allergenic spores and by causing spoilage. To improve storage techniques and to evaluate products with respect to their level of fungal contamination, reliable methods for quantifying fungal infection are urgently needed.

Food-borne fungi are often quantified by counting CFU (5), although it is generally recognized that this method provides a poor estimate of fungal biomass (14). Alternative techniques, based on differences in chemical composition between fungi and their substrata, have been developed to replace the traditional plate counts. One such method is the quantification of ergosterol (17), a fungus-specific membrane lipid (19). The ergosterol assay has become widely used for estimating the degree of fungal contamination of grains and other plant materials.

New quantitative methods are often assessed by comparing the results of these methods with CFU counts. Ideally, these methods should also be evaluated against a more absolute biomass measure, such as fungal dry weight. However, with the exception of agar substrates (10), this is not possible when fungi are growing in a solid substrate. The only other alternative is to determine fungal volume through microscopical determinations of hyphal length and spore numbers in a homogenate.

In this study, the ergosterol assay has been evaluated by comparing its results with hyphal length measurements and CFU counts. As test organisms, three food-borne fungi with different growth patterns were chosen and grown on a synthetic agar substrate. A second objective was to quantify the relative contributions of hyphae and spores to total fungal biomass.

MATERIALS AND METHODS

Organisms. The different fungal strains were from our own culture collection. *Fusarium culmorum* (W. G. Smith) Sacc. (J-15) was grown on agar slants of Syntetischer Nährstoffarmer agar (8), and *Penicillium rugulosum* Thom (J-1) and *Rhizopus stolonifer* (Ehrenb.) Lind (J-45) were grown on agar slants containing 2% malt extract (Oxoid).

Substrate and inoculation. The substrate, modified from the one developed by B. Norkrans (9), contained the following per liter: 10.0 g of glucose, 1.1 g of $NH_4H_2PO_4$, 0.33 g of KH_2PO_4, 0.67 g of K_2HPO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 114 mg of $CaCl_2 \cdot 2H_2O$, 10 mg of $C_6H_5O_7Fe \cdot 5H_2O$ (ferric citrate), 4 mg of $ZnSO_4 \cdot 7H_2O$, 4 mg of $MnSO_4 \cdot 4H_2O$, and 1 mg of $CoCl_2$. In the preparation of agar plates, 15 g of agar (Oxoid) per liter of medium was added. The final pH was 6.3. Spores were harvested from agar slants by adding sterile tap water with 0.02% Tween 80. A 20-μl portion of inoculum, containing ca. 5×10^3 spores, was placed in the center of a petri dish (inner diameter, 87 mm) containing 20 ml of the agar containing glucose and mineral salts.

Growth measurements. Fungal growth was quantified in terms of radial growth rate, number of CFU, and ergosterol content and by microscopical determinations of hyphal length and spore number. For CFU determinations, the colonies were cut into pieces (5 by 5 mm) and shaken with tap water containing 0.02% Tween 80 for 30 min on a rotary shaker at 250 rpm. Suitable dilutions were spread on the surface of agar containing 2% malt extract (Oxoid). CFU on the plates were counted after incubation for 3 days at 24°C.

Ergosterol in the agar plates was extracted by 2 h of refluxing with methanol, followed by the addition of KOH in ethanol to provide a saponification step during 30 min of further refluxing (15). Ergosterol was analyzed using a Waters high-pressure liquid chromatography system with a Novapak C_{18} column and with methanol (2 ml/min) as the mobile phase. The presence of ergosterol was detected at 280 nm about 8 min after injection.

Hyphal lengths were estimated by direct microscopy on agar slides, essentially as described earlier (13). The agar was cut into pieces (5 by 5 mm) and homogenized in sterile tap water for 60 s with an Ultra-Turrax at top speed (16,000 rpm). After appropriate dilutions in tap water, 1 ml of homogenate was added to 1 ml of melted 1.5% agar (Difco). A 45-μl portion of this mixture was placed on a warm microscope slide. A coverslip (22 by 26 mm) was then placed

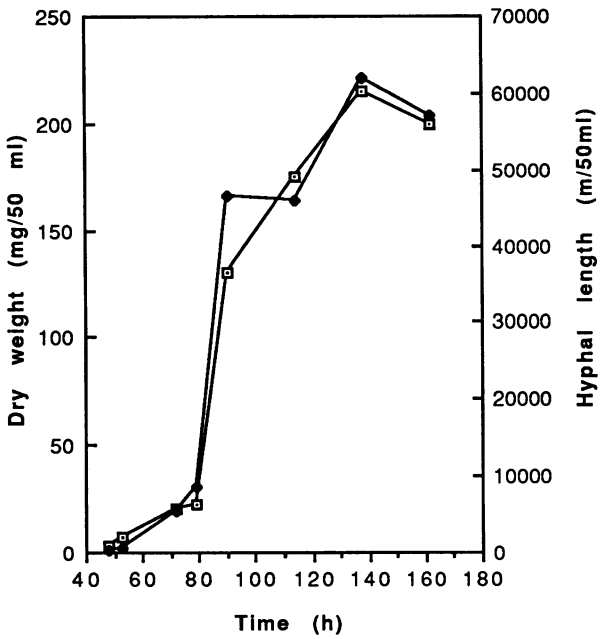

FIG. 1. Comparison of dry weight (⊡) and hyphal length (◆) determinations during growth of *P. rugulosum* in liquid culture. Each datum point represents the mean of three replicates. The average coefficient of variation was less than 10%. m, meter.

on top of the still fluid agar, which precisely filled the area beneath the coverslip. The slides were examined at ×400 magnification, and hyphal lengths were measured using the intersection technique (12). The same technique was used to estimate hyphal lengths in homogenates from liquid cultures. Spore numbers were quantified in a hemocytometer.

All values are expressed as means per agar plate from three replicates. The average coefficient of variation was less than 10%.

Biomass calculations. Fungal biomass was calculated from measured hyphal lengths and diameters and from measured spore numbers and diameters, assuming densities of 1.09 g cm^{-3} and 21% dry weight (1).

RESULTS

Visual observations of fungal growth. The three fungi showed very different growth patterns. *R. stolonifer* grew rapidly (18 mm day^{-1}), produced many spores, and reached the edge of the agar plate after 60 h. *F. culmorum* grew as a floccose, cottony mycelium at 7.6 mm day^{-1} and did not produce spores. *P. rugulosum* grew as a dense colony at 1.5 mm day^{-1} and produced many spores.

Fungal growth measurements. To evaluate the method for hyphal length measurements, *P. rugulosum* was grown in liquid culture using the modified Norkrans medium described above. Both the dry weight and hyphal length of the fungal biomass were measured. The resulting growth curves were almost identical for the two different measurements (Fig. 1). This experiment made it possible to compare calculated and measured mass-to-length ratios. On the basis of data from samples taken at 116 h, the measured ratio was 4.2 μg m^{-1}, while conversion from hyphal length and diameter (assuming a density of 1.09 g cm^{-3} and a dry weight of 21%) gave a ratio of 6.7 μg m^{-1}.

Hyphal lengths and ergosterol levels increased in parallel

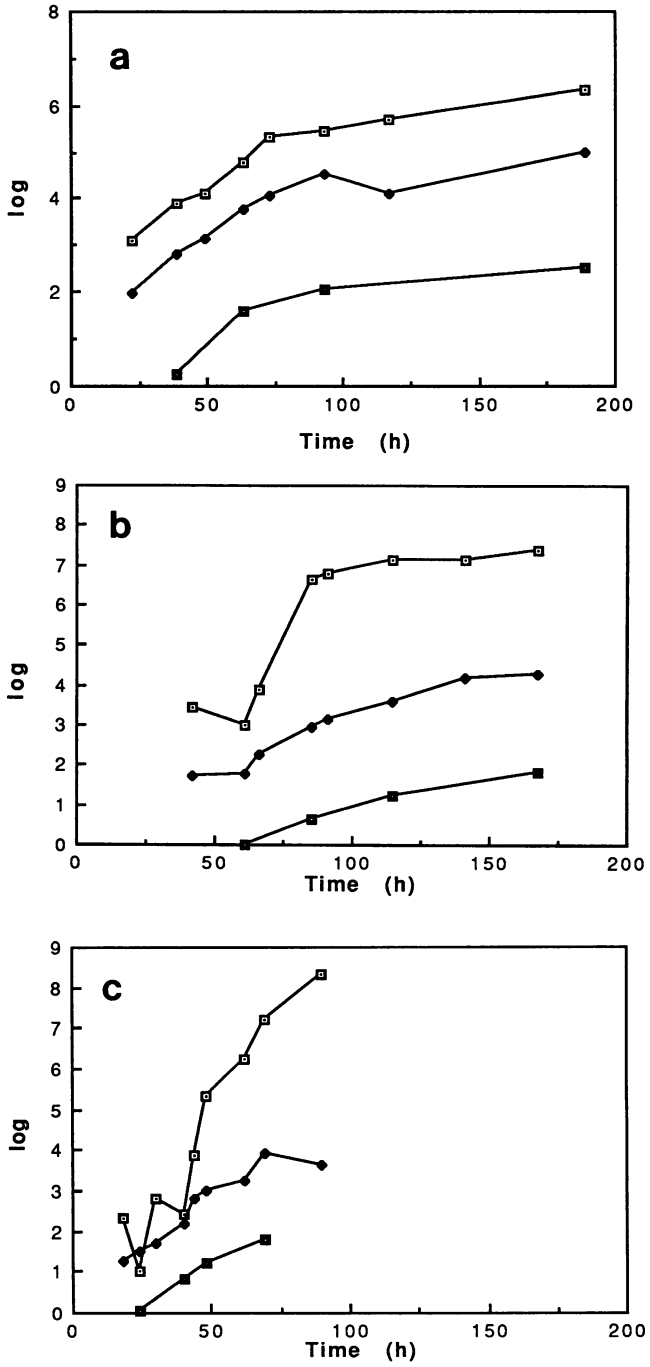

FIG. 2. Growth of *F. culmorum* (a), *P. rugulosum* (b), and *R. stolonifer* (c) on agar containing glucose and mineral salts measured in CFU (⊡), hyphal length (in meters $plate^{-1}$) (◆), and ergosterol content (in micrograms $plate^{-1}$) (■). All values are given as log units and represent the means of three replicates. The average coefficient of variation was less than 10%.

for all three fungi on the agar substrate (Fig. 2). CFU values followed increases in hyphal length and ergosterol content for the nonsporulating *F. culmorum* (Fig. 2a) but not for the other two fungi (Fig. 2b and c). The production of spores, as observed by direct microscopy (data not shown), coincided with drastic increases in numbers of CFU. With *P. rugulo-*

TABLE 1. Summary of biomass measurements for *F. culmorum* (285 h), *P. rugulosum* (234 h), and *R. stolonifer* (162 h)[a]

Fungus	No. of CFU	Hyphal length (m)	Hyphal mass[b] (mg)	No. of spores	Spore mass[b] (mg)	Fungal mass[b] (mg)	Ergosterol content	
							μg	% of fungal mass (dry wt)
F. culmorum	6×10^6	54,000	307	0	0	307	418	0.14
P. rugulosum	2×10^8	21,000	76	1×10^9	4	80	110	0.14
R. stolonifer	2×10^8	10,000	338	2×10^8	10	348	129	0.04

[a] All values are given per agar plate and are the means of three replicates.
[b] Dry weight calculated from measured fungal volumes.

sum, ergosterol increased 4-fold between 62 and 85 h postinoculation whereas the corresponding increase in CFU values was 4,600-fold. Similar results were observed for *R. stolonifer*. Once the sporulation phase had ended, the relative increases recorded with the two methods of biomass measurements were comparable.

Later (not shown in Fig. 2), samples were taken for a final biomass determination (Table 1). The CFU values for *P. rugulosum* were almost 2 orders of magnitude higher than those of *F. culmorum*, but its biomass was only one-fourth. For the two sporulating fungi, the numbers of spores counted with the microscope were of the same order of magnitude as CFU counts. Conversions to dry weights showed that spores contributed 3 to 5% to total fungal mass. Ergosterol contents were between 0.04 and 0.14% of the calculated fungal mass (dry weight).

DISCUSSION

New immunological methods have made it possible to quantify soluble antigens from certain fungal species or genera (4, 6). However, a method able to measure the total fungal biomass in a sample is also needed. At present, the CFU count is the most commonly used method in industry and in research. However, although simple to perform, this method is time-consuming and serious doubts exist as to whether it gives a reliable quantitative measure of the extent of fungal infection (14). In this investigation, CFU counts were found to vary greatly, depending on whether fungal sporulation had occurred. Since the spore mass constituted only a small percentage of the total fungal mass, periodic CFU counts will not accurately reflect changes in fungal biomass.

Chitin, a cell wall polymer of *N*-acetyl-D-glucosamine, and ergosterol, a fungus-specific membrane lipid, are found in ascomycetes, zygomycetes, deuteromycetes and in most basidiomycetes, i.e., in all food-spoiling and mycotoxin-producing molds (2, 19, 20) and thus should be good candidates for analyses. The quantification of chitin is time-consuming, but the more rapid measurement of ergosterol has gained wide acceptance (7, 15, 21, 22), especially for use with cereals. We reported earlier that increases in ergosterol correlated well with those of volatile fungal metabolites and CO_2 production but not with increases in CFU (3).

The ergosterol content of fungal mycelium, as determined in the present investigation, was slightly lower than that found when fungal mass was quantified gravimetrically. Seitz et al. (18) found that ergosterol contents ranged from 0.2 to 0.6% for *Aspergillus flavus*, *Alternaria alternata*, and *Aspergillus amstelodami*. Using a gravimetric method for quantifying fungal mass, Nout et al. (11) determined that the ergosterol content of *Rhizopus oligosporus* when grown in synthetic laboratory medium varied with age from 0.2% in young cultures to 2.4% for old ones. The observed increase in ergosterol content with age was probably due to an ageing-associated vacuolization of the mycelium (16). This vacuolization would lead to a reduction in weight per unit of length and, consequently, to an increased ergosterol-to-mass ratio. When *R. oligosporus* was grown on a solid substrate (soybeans) and when the fungal mass was quantified by direct microscopy, the ergosterol content varied between 6 and 9% (11). The higher ergosterol concentrations obtained in solid substrates compared with those obtained in liquid culture might have resulted from errors in hyphal length measurements and from uncertainties with regard to conversion factors. These investigators also found that a reduction in oxygen tension reduced the ergosterol content. The effects of a reduced oxygen supply on the ergosterol level of food-contaminating fungi need further investigation, especially in relation to fungal growth in natural substrates. In the present study, it was found that *R. stolonifer* had the lowest ergosterol content of the three species investigated (0.04%). This species also had the widest hyphal diameter; consequently, any incorrect assumptions used in the conversion from length to mass would have led to a proportionally higher mass than for the other two species.

Thus, differences in growth patterns between different fungal species, methodological problems, and environmental effects make it futile to search for a fixed conversion ratio for ergosterol level to biomass. At this time, there is no ideal method for quantifying fungal growth in solid substrates. Nevertheless, this investigation has shown that for three fungi with widely different growth patterns, changes in ergosterol content accurately reflect changes in biomass, as estimated by hyphal length, whereas mold colony counts (CFU) are, to a large extent, a measure of sporulation.

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