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Growth-Promoting Effect of Thermophilic Fungi on the Mycelium of the Edible Mushroom *Agaricus bisporus*

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The growth-promoting effect of the thermophilic fungus *Scytalidium thermophilum* in mushroom compost on the mycelium of the edible mushroom *Agaricus bisporus* was investigated. Results obtained by others were confirmed by showing that *S. thermophilum* leads to an increased hyphal extension rate of the mushroom mycelium. However, it was demonstrated that hyphal extension rates were not clearly related to mushroom biomass increase rates. A number of experiments pointed strongly towards CO_2 as the determinant of hyphal extension rates. In compost, CO_2 is produced mainly by thermophilic fungi. Several experiments did not reveal any other specific compound produced by *S. thermophilum* that increases the hyphal extension rate of the mushroom mycelium.

The production of compost for the cultivation of the edible mushroom *Agaricus bisporus* consists of a 10-day uncontrolled outdoor composting process followed by an indoor, temperature-controlled process (7, 22). During the indoor phase a temperature of 56 to 58°C is maintained for 12 to 20 h, followed by a 4- to 5-day period at 46 to 48°C (7).

Thermophilic fungi, defined as exerting growth at temperatures exceeding 40°C (3), grow massively during the last phase of the indoor process (19, 26) and contribute significantly to the quality of the compost (14, 15, 19). They are considered important because they are beneficial for the selective growth of *A. bisporus*, first by decreasing the ammonia concentration (15) and second by immobilizing nutrients (5). Third, they have a positive effect on the extension rate of the mushroom mycelium, as has been demonstrated for *Scytalidium thermophilum* and several other thermophilic fungi (19, 20).

This growth-promoting effect of *S. thermophilum* on *A. bisporus* is intriguing, because until now no satisfactory explanation was available. The effect has been observed whether the *S. thermophilum* mycelium was alive or dead (20). Also, the frequency of *S. thermophilum* propagules in mushroom compost has been observed to be positively correlated with the mushroom yield from the compost (19).

It was implied from these observations that the thermophilic fungi produce or contain a specific compound promoting the growth of *A. bisporus*. However, growth promotion of *A. bisporus* by a specific compound apart from CO_2 (16) has not been reported to date.

We have started an investigation into the nature of the growth promotion. The problem was approached by quantifying growth of *A. bisporus* under various conditions, not only on the basis of mycelial extension rates but also on the basis of CO_2 production rates and laccase excretion. Laccase is an extracellular polyphenoloxidase possibly involved in lignin breakdown (28). The laccase content of compost can be used as a measure for the amount of mushroom mycelium present in that compost (11, 27, 29). Mycelial extension is not necessarily correlated with biomass increase (2), whereas CO_2 production and laccase excretion are directly related to biomass increases.

MATERIALS AND METHODS

Organisms. *S. thermophilum* 15.8 was obtained from the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands. The commercial *A. bisporus* strain X1 (Le Lion, Zeddam, The Netherlands), pregrown axenically on wheat grains, was used in all experiments with mushroom mycelium.

Media and growth conditions. *S. thermophilum* was routinely maintained on solid media containing, per liter, glucose, 10 g; yeast extract, 5 g; and agar, 15 g. Liquid cultures were grown on a medium containing, per liter, glucose, 10 g; sodium glutamate, 0.135 g; KCl, 0.2 g; $MgSO_4$, 0.2 g; $CaCl_2$, 0.2 g; and yeast extract, 0.1 g, supplied with 1 ml of a trace element solution (24). The fungus was grown at 45°C. It was harvested from the culture medium by filtration through sterile cheesecloth carried out in a laminar flow hood. Cell extracts were prepared by freeze pressing harvested cells in a prechilled X-Press (Biox, Göteborg, Sweden). Cell extract was obtained by centrifugation at 30,000 × *g* for 20 min at 4°C.

A. bisporus X1 was grown routinely on malt extract agar (Difco) plates. For inoculation in composts, commercial spawn was used. Inoculation on plates was done by using plugs (diameter, 5 mm) from plates with mycelium to replace plugs on sterile plates. Incubation with *A. bisporus* took place at 23 to 24°C.

Compost. Starting material for the production of mushroom compost (consisting of a mixture of horse manure, straw, chicken manure, gypsum, and water) and fully prepared mushroom compost were obtained from a commercial compost factory, Gebr. Theeuwen B.V. in Blitterswijck, The Netherlands.

The materials were sterilized by autoclaving twice at 120°C for 20 min on two separate days with an incubation at 45°C for 4 h in between. After sterilization, sterile water was added to account for the weight loss by evaporation. No further additions were made.

Water extracts of mushroom compost were made by 1:1 (wt/wt) addition of water to compost, filtration through

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cheesecloth, and centrifugation twice at 37,000 × *g*. This extract was filter sterilized before use.

Mycelial growth rate tests. Tests for the determination of the growth rate of the mushroom mycelium in composts were carried out in glass cylinders (18 by 2.75 cm), hereafter referred to as test tubes, stoppered with cotton plugs at both sides, with 30 g of compost and 20 grains of mushroom spawn (19, 23). In some experiments, one such test tube was connected with an identical test tube filled with nonsterile mushroom compost. To this end a single 2-cm cotton plug was inserted to connect the tubes and separate the materials, and the connection was sealed with the adhesive tape. Inoculation of the sterile compost with mushroom mycelium was then carried out at the nonconnected end of the test tube.

Experiments in which the CO_2 concentration in a test tube was controlled were carried out by passing either air or air with 0.4% CO_2 at a rate of 0.5 liter/min through compost test tubes. The gas passed through a 10-liter bottle with water at 40°C and two 1-liter Erlenmeyer flasks at 24°C to achieve water saturation of the gas, and an 18-cm test tube was filled with sterile cotton to prevent contamination of the compost test tubes.

CO_2 accumulation measurements. The CO_2 accumulation of various compost test tubes was measured by keeping the test tubes in 6-liter desiccators. Periodically samples were withdrawn for analysis. If the CO_2 concentration in the desiccators exceeded 8%, the desiccator content was flushed with air.

For the measurement of the radial extension rate of colonies on agar plates as a function of the CO_2 concentration, the agar plates were kept in 6-liter desiccators. The CO_2 concentration in the desiccators was measured daily, and whenever it had changed, the desiccator content was flushed with air or N_2-O_2 and brought to the original concentration.

Activity tests. Activity of biomass present in the compost was measured by an adapted respiration test (25). Glucose and nutrients were added to small compost samples, and the evolution of CO_2 was monitored over time. Nystatin (15 mg/g of compost) was used to prevent fungal activity, and streptomycin (15 mg/g of compost) was used to prevent bacterial activity.

Laccase measurements. The concentration of laccase was measured in 200-ml cotton-stoppered Erlenmeyer flasks containing 10 g of (sterile) compost. The compost was inoculated with 7 grains of mushroom spawn. For each determination, a separate Erlenmeyer flask was used.

A qualitative measure for the laccase concentration in agar plates was obtained after extraction of laccase in the plates with 100 ml of 0.01 M phosphate buffer, pH 7.0, for 24 h at 4°C.

Laccase was assayed by a combination of methods (9, 10, 27, 29). A 0.01 M phosphate buffer, pH 7.0 (20 ml), was added to 10 g of compost. Extraction took place on a rotary shaking incubator at 25°C for 30 min. The extract was filtered through muslin and centrifuged for 20 min at 4°C at 37,000 × *g*. Extracts were measured either directly or after storage at −20°C. Laccase activity was measured at 30°C in a mixture containing 1.5 ml of 0.02 M sodium acetate buffer (pH 5.0), 0.2 ml of 0.5 mM syringaldazin in ethanol, and maximally 0.2 ml of extract in a 1.9-ml total volume. The increase in extinction at 525 nm was monitored, and the slope of the line was taken as a measure for the laccase activity. The molar extinction coefficient of the product of the reaction of laccase with syringaldazin (tetramethoxy-azo-bis-methylene quinone) was 75,000 $M^{-1} \cdot cm^{-1}$ under the test conditions employed. One laccase unit is defined as the amount of enzyme producing 1 μmol of the product of the reaction with syringaldazin min^{-1}.

Analyses. Determinations of concentrations of CO_2 were made with a Becker 427 gas chromatograph equipped with a thermal conductivity detector. A Hayesep Q column (2 m by 2 mm) with helium as the carrier gas (ca. 17 ml/min) was used. Temperatures of the oven, injector, and detector were 110, 110, and 240°C, respectively. The dry weight of samples of fungal mycelium and compost was measured after drying to a constant weight at 70°C.

RESULTS

Growth measurements. The growth-promoting effect of *S. thermophilum* on *A. bisporus* was easily demonstrated in compost tube tests. The rate of extension of the mushroom mycelium was twofold greater either in mushroom compost or in sterile mushroom compost pregrown with *S. thermophilum* than in sterile compost (Fig. 1A). However, accumulation of CO_2, as measured in closed 6-liter vessels, proceeded in sterile compost as fast as in *S. thermophilum*-preincubated sterile compost. It occurred at approximately 65% of the rate observed in mushroom compost inoculated with *A. bisporus* (Fig. 1B). The increase of biomass of *A. bisporus*, assessed by measuring the laccase concentration in compost, was the same in sterile and nonsterile mushroom compost. In the sterile compost, however, a significant lag phase was apparent (Fig. 1C). This long lag phase was due to the low CO_2 concentration in the sterile compost, as is explained below.

Effect of *S. thermophilum* on mycelial extension rate of *A. bisporus*. Subsequently, whether the *S. thermophilum* mycelium itself or a specific metabolite affected the mycelial extension rate of *A. bisporus* was investigated. Mycelium of *S. thermophilum* added aseptically to sterile compost at 5% (wt/wt) had the same effect on the extension rate of the mushroom mycelium as preincubation had. Addition of 5% (wt/wt) *S. thermophilum* mycelium to sterile starting material for the production of mushroom compost led to approximately the same rate of mycelial extension, albeit only after a considerable lag period (presumably due to slow NH_3 removal [15]). Sterile starting material itself failed to give rise to any growth of the mushroom mycelium. These experiments are summarized in Fig. 2.

No increase of the mycelial growth rate was observed in compost test tube experiments in which the supernatant, pellet, and both components after cell disruption and centrifugation of the mycelium of *S. thermophilum* were added to sterile compost. Also, spent medium after growth of the fungus or a mixture of the vitamins niacin, thiamine, biotin, and pantothenic acid (23) did not affect the growth rate of the mushroom mycelium. A water extract of compost, which has a moderately positive effect (increase in radial extension rate from 2.3 to 3.3 mm/day) on the extension rate on agar plates (see also reference 12), had no effect in compost tube tests (data not shown).

In a number of experiments *S. thermophilum* mycelium was pregrown in compost and subsequently killed, after which *A. bisporus* was inoculated in compost tube tests. No growth-promoting effect was observed, whether the *S. thermophilum* mycelium was killed by heat sterilization, ethylene oxide treatment, irradiation, or pasteurization for 4 h at 70°C (data not shown).

Increase in extension rate by gas produced by compost. In test tubes with sterile mushroom compost which were connected to similar tubes containing unsterilized mushroom

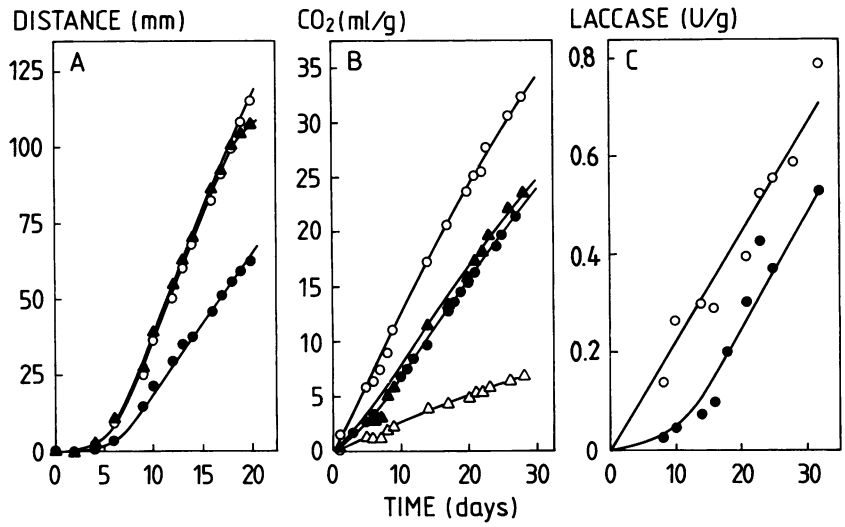

FIG. 1. Comparison of different measures of growth of *A. bisporus* on various composts. (A) Distance from the inoculation point as a function of time in compost tube tests; (B) CO_2 evolution from compost tubes as a function of time; (C) laccase activity in compost as a function of time in composts. Substrates for growth of *A. bisporus*: ○, mushroom compost; ▲, sterile mushroom compost, preincubated with *S. thermophilum*; ●, sterile mushroom compost. △, *S. thermophilum*-preincubated compost without *A. bisporus*.

compost, *A. bisporus* showed a mycelial extension rate 33% higher than that in tubes not connected (Fig. 3). Although small, this difference was consistently reproducible. After growth of the mushroom mycelium for 22 days, the CO_2 concentration at a depth of 2 to 3 cm from the inoculation side was 0.33% ± 0.07% in tubes connected with tubes of unsterilized compost, as opposed to 0.11% ± 0.02% in similar but detached tubes. Mushroom compost with mushroom mycelium growing for 20 days had a CO_2 concentration of 0.41% at a 2- to 3-cm depth.

Compost itself produced CO_2 at 24°C nearly linearly for up to a month at a rate of 0.81 ml · g^{-1} · day^{-1}. Sterile compost pregrown for 5 days at 45°C with *S. thermophilum* produced 0.27 ml · g^{-1} · day^{-1} at 24°C at a virtually constant rate for at least 25 days.

Influence of CO_2. In Fig. 4 the hyphal growth rate of the mushroom mycelium on various solid media is presented as a function of the CO_2 concentration. This growth rate was constant for at least 16 days after an initial 5-day lag phase. Apparently the CO_2 concentration has a strong influence on the hyphal extension rate: on three different solid media, the hyphal extension rate increases until CO_2 concentrations of approximately 0.5% are reached, above which concentration the hyphal extension rate slowly levels off. The values of the highest hyphal extension rates on agar plates were typically two to three times lower than those in normal compost (5,

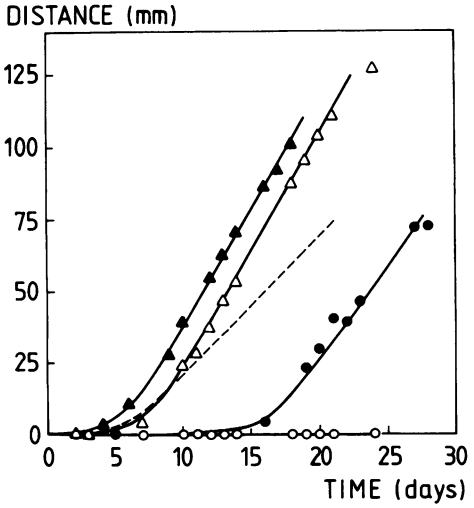

FIG. 2. Extension of *A. bisporus* mycelium in compost tube tests with various pretreated composting materials. ▲, sterile mushroom compost preincubated with *S. thermophilum*; △, sterile mushroom compost with 5% (wt/wt) mycelium of *S. thermophilum* pregrown in liquid culture added; ●, sterile starting material with *S. thermophilum* from liquid culture added; ○, sterile starting material. The dotted line represents extension in sterile compost as in Fig. 1A.

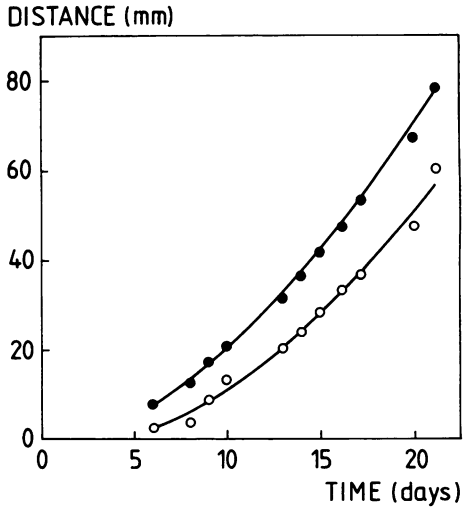

FIG. 3. Extension of *A. bisporus* mycelium in compost tube tests with sterile composting material, with (●) or without (○) connection to a test tube with nonsterile mushroom compost.

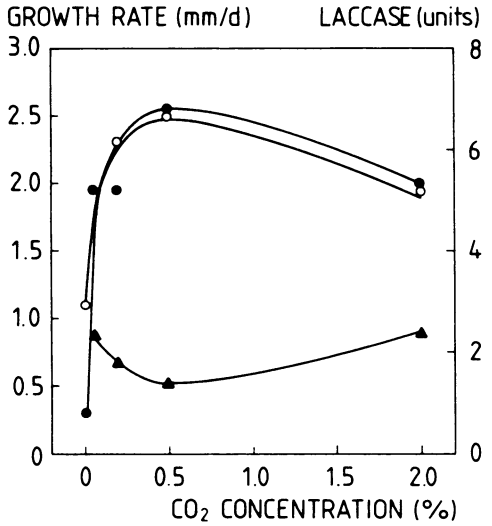

FIG. 4. Radial extension rate of mycelium of *A. bisporus* as a function of the CO_2 concentration. ●, growth rate on malt extract agar, 40 g/liter; ○, growth rate on compost extract agar; ▲, laccase content (in arbitrary units) of the malt extract agar plates after 20 days of growth.

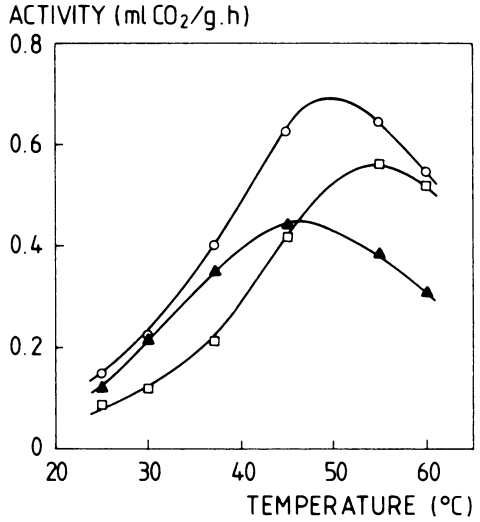

FIG. 6. Respiration of samples of mushroom compost as a function of temperature. ○, no additions; ▲, with addition of streptomycin; □, with addition of nystatin. Datum points are the averages of five determinations.

12, 13). At the end of the experiment, the laccase contents of the colonies on the malt extract agar plates were measured. They showed no linear or quadratic correlation with the colony diameter (Fig. 4).

On the basis of the observed effect of CO_2, whether varying the CO_2 concentration in compost test tubes influenced the hyphal extension rate was tested. Purging compost test tubes with air with various fixed CO_2 concentrations revealed an effect of CO_2 on the mycelium extension rate for both normal and sterile compost (Fig. 5). In sterile compost, the linear hyphal extension rate at 0.04% CO_2 was 3.1 mm/day as opposed to 5.7 mm/day at 0.4% CO_2. In normal compost, these values were 4.4 and 6.4 mm/day, respectively.

In another experiment, either sterile compost or sterile compost pregrown with *S. thermophilum* was used. The CO_2 concentration upon incubation with *A. bisporus* was maintained at 0.4%. Mycelial extension rates in both systems were similar (data not shown).

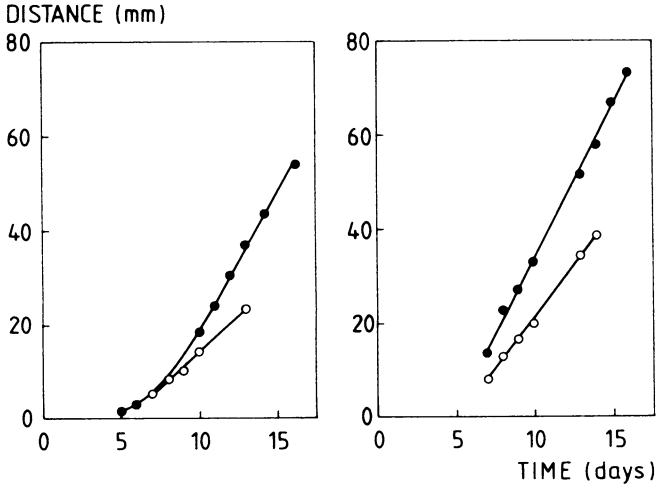

FIG. 5. Extension of *A. bisporus* mycelium in compost tube tests with various CO_2 concentrations. ○, 0.04% CO_2; ●, 0.4% CO_2. (Left) Sterile compost; (right) normal compost.

Respiratory activity tests. Both bacteria and thermophilic fungi are numerous in mushroom compost. This raises the question of why thermophilic fungi and not bacteria would be responsible for the increase in the extension rate of *A. bisporus*. Therefore, the activities and the temperature optima of both groups of organisms were estimated. The activity of the compost without an addition and after addition of either streptomycin or nystatin was measured by respiration tests. In Fig. 6 the respiration in mushroom compost is presented as a function of temperature. The three curves show three different temperature optima. As streptomycin is active mainly against prokaryotes, the residual respiration after its addition will be caused by fungi, as all other eukaryotes will be killed off during the production process of the compost. As is clear from Fig. 6, the residual activity after streptomycin addition is nearly 100% of the total activity in the lower-temperature region, whereas at the higher temperatures tested, the relative contribution of streptomycin-inhibited respiration decreases strongly. The residual activity after addition of the antifungal antibiotic nystatin, however, is low in the lower-temperature region but increases strongly in the higher temperature range. This indicates widely differing temperature optima for the bacteria and the fungi in the compost.

DISCUSSION

There can be little doubt that thermophilic fungi play a key role in the preparation of mushroom compost and that they have a positive effect on the quality of the compost (Fig. 2).

However, what the exact positive effect of the thermophilic fungi on the growth rate of *A. bisporus* is must be considered quite unclear. It is clear that the presence of *S. thermophilum* in the compost generates a mycelial extension rate which is much greater than the rate in sterile compost. In our experiments, increased mycelial extension rates of *A.*

bisporus have not been observed in compost containing killed *S. thermophilum* mycelium. This is at variance with reports by Straatsma et al. (20). Nor did we find any increase in the extension rate of *A. bisporus* when cell extracts or cell components of *S. thermophilum*, spent medium, or even water extracts of mushroom compost were added to sterile mushroom compost.

In our hands, it seems that the increase in the hyphal growth rate was caused by enhanced levels of CO_2 (Fig. 3 and 5). A positive effect of CO_2 concentrations of up to 1% in air on the mycelial growth rate of the mushroom mycelium has been demonstrated previously (16). Apparently the mushroom mycelium encounters optimal, or nearly optimal, CO_2 concentrations during a tube test.

With sterile compost, however, the CO_2 production by the mushroom mycelium itself is not enough to maintain CO_2 levels in the stimulatory range. Under these conditions, CO_2 concentrations were much lower than the optimum CO_2 concentration for growth. Apparently the CO_2 concentration in the compost is the primary factor determining the hyphal extension rate.

Accumulation of CO_2 in closed systems, reaching a concentration of 1% within 4 days, explains the high growth rate during growth of *A. bisporus* in sterile compost compared with the growth rate in sterile compost pregrown with *S. thermophilum* (Fig. 1B). The ventilation as a result of incubation in cotton-plugged Erlenmeyer flasks apparently generates a low CO_2 concentration and thus a lag phase in the biomass increase rate (Fig. 1C). The laccase measurements show that the biomass increase rate in sterile compost is as high as in normal compost. From this observation and also from the lack of correlation between laccase concentration and colony diameter on agar plates, it can be concluded that in *A. bisporus*, the hyphal extension rate and biomass increase rate are not clearly related.

It must be noted, though, that extension rates of 8 to 9 mm/day, as found in normal compost, were not achieved during most of our tests with sterile composts at high CO_2 concentrations (Fig. 5). Therefore, it cannot be ruled out that the *S. thermophilum* mycelium itself contains or excretes as yet undetectable additional compounds that stimulate the extension of the mushroom mycelium.

From the production scheme of mushroom compost, it may be explained that thermophilic fungi, not bacteria or actinomycetes, in the compost generate the growth-promoting CO_2. Most mesophilic bacteria will not survive the first high-temperature phase or the pasteurization phase. During cultivation of the mushroom mycelium, mesophilic bacteria will grow only sparsely (8) because of the lack of suitable substrates. As a result, 50 to 70% of the biomass of the compost at the end of the production process consists of thermophilic fungi (17, 25). Some of these thermophilic fungi are still active at lower temperatures, down to 25°C: 15 of 20 taxa tested showed growth at this temperature (13). Thermophilic or thermotolerant bacteria consist in large part of *Bacillus* species (21). Generally, these bacteria will exert a very low activity at 24°C, the temperature at which the mushroom mycelium is grown. The measurements presented in Fig. 6 corroborate this hypothesis. The temperature at which the mushroom mycelium is grown, 24°C, is about 30°C from the temperature optimum of the bacteria and only 20°C from that of the fungi. It may be clear that the CO_2 generated by compost during the growth of the mushroom mycelium is generated in large part by thermophilic fungi.

Mycelia in general tend to "search" (1) for their substrate. At low substrate concentrations sometimes high mycelial extension rates can be found. Apparently CO_2 concentrations in the range of 0.3 to 1.0% generate a higher mycelial extension rate without affecting the biomass increase rate. This high extension rate may be the response to sensing the presence of other biomass by the CO_2 concentration. Thus, high hyphal extension rates may, in *A. bisporus*, have an ecological significance: it may be useful to grow as fast as possible, thereby colonizing as much substrate as possible. Once the substrate has been occupied, the mycelium seems to be able to prevent the occupation by other microorganisms, either by consuming them (5, 6) or by excretion of CO (18), which effectively inhibits growth of most competing organisms but inhibits the growth of the mushroom mycelium itself only partly (4).

It can be concluded that thermophilic fungi, although important in the production of mushroom compost, seem not to have a specific growth-stimulatory effect on the mushroom mycelium. It is the CO_2 production by the thermophilic fungi that leads to the observed higher growth rate. This higher growth rate itself, however, is only an effect of a higher rate of hyphal extension. It does not coincide with a higher rate of biomass increase of the mushroom mycelium. It may have ecological significance, though.

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