Significance of thermophilic fungi in mushroom compost preparation: effect on growth and yield of *Agaricus bisporus* (Lange) Sing.

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Eighteen species of thermophilic and thermotolerant fungi were isolated from mushroom compost. Growth of *Agaricus bisporus,* was studied on sterile compost pre-colonized with four thermophilic fungi *viz*., *Chaetomium thermophile, Malbranchea sulfurea, Thermomyces lanuginosus* and *Torula thermophila.* All the four fungi were inoculated singly and in different combinations on sterilized compost to evaluate their potential to promote growth and yield of *A. bisporus*. A mixed inoculum of *Malbranchea sulfurea* and *Torula thermophila* was found to be the best amongst the various treatments that promoted the growth of *A. bisporus* to the plateau of 7.7 mm day^{-1} and the yield of the mushroom was almost twice compared to the pasteurized control. The effect of *T. lanuginosus* when inoculated singly or in combination with other thermophilic fungus/fungi in compost was insignificant resulting in lower growth rates. Mycelial extension rate, pH of the compost before spawning, yield of mushrooms and biological efficiency for various treatments were studied. The study reveals that thermophilic fungi provide for compost selectivity and protection against negative effects of compost bacteria on mycelial growth of *A. bisporus.* This finding is of relevance for the commercial production of high-yielding mushroom compost for *A. bisporus.*

Key Words: biological efficiency, crop yield, growth rate, mixed culture

Introduction

The white button mushroom (*Agaricus bisporus*) (Lange) Sing. is cultivated on a substrate consisting of a composted mixture of straw bedded horse manure, wheat straw, chicken manure and gypsum. Compost is prepared in a sequence of processes. Conventionally two phases of composting are distinguished (Sinden and Hauser, 1950; Fermor *et al*., 1985). After mixing and moistening the ingredients, the mixture is left for a short period and

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subjected to phase I composting process in the open air. During phase-I NH_3 and unpleasant smelling compounds are emitted into the environment. It is followed by phase-II: an indoor, temperature-controlled process. Subsequently, conditioning of the compost is carried out at approximately 45°C. This can either be performed in limited quantities in mushroom houses or in bulk in 'tunnels. In mushroom houses self-heating of the compost is controlled by ventilating air around layers of up to 30 cm thick contained in trays or polyethylene bags. Whereas in tunnels fresh air is forced through the body of the compost, allowing an accurate control, and layers up to 250 cm can be processed (Derks, 1973; Gerrits 1988a). Several other investigators suggested that combined phase-I and phase-II indoor composting is feasible (Laborde *et al*., 1987; Perrin and Gaze, 1987; Gerrits, 1987; Straatsma *et al*., 1989, 1991, 1994a & b; Wiegant, 1992).

Thermophilic fungi are believed to contribute significantly to the quality of compost (Seal and Eggins, 1976; Eicker, 1977; Ross and Harris, 1983; Gerrits, 1988b). The effect of these fungi on the growth of mushroom mycelia and mushroom yield have been described at three distinct levels (Wiegant, 1992). First, they decrease the concentration of ammonia in the compost, which otherwise would counteract the growth of the mushroom mycelium. Second, they immobilize nutrients in a form that apparently is available to the mushroom mycelia. And third, they may have a growth promoting effect on the mushroom mycelia, as has been demonstrated for *Scytalidium thermophilum* and for several other thermophilic fungi (Wiegant *et al*., 1992). The effectiveness of *S. thermophilum* in compost preparation for *A. bisporus* has been shown by Straatsma *et al*. (1994a) which obtained a two folds increase in the yield of mushrooms on inoculated compost when compared to the pasteurized control.

In order to produce compost with constant high quality that does not emit ammonia and odour into the environment, the artificial inoculation and controlled preparation of compost is highly desirable. This study focuses on the significance of thermophilic fungi in reducing the time for compost preparation and their effect on growth rate and crop yield of *A. bisporus* by single and dual culture inoculation.

Materials and methods

Substrates

The formulation used for compost preparation for the cultivation of white button mushroom consisted of wheat straw (chopped 8-20 cm long), 300 kg;

wheat bran, 15 kg; chicken manure, 125 kg; urea, 5.5 kg; gypsum, 20 kg and BHC (10%), 125 g. Ingredients of the compost were obtained from the local market. Traditional scheme of mixing and moistening the ingredients for compost preparation were applied. Here the term 'young compost' is used for any substrate prior to phase-II. The growth of thermophilic fungi was tested on young compost that was obtained from a local mushroom farmer at Kurukshetra.

Isolation of thermophilic fungi

One kg of compost was randomly sampled in 100 g portions, mixed thoroughly and was used for isolation. Thermophilic fungi were isolated from different composting phases by using serial dilution method on YpSs agar (Yeast extract, 4.0 g; K_2HPO_4, 1.0 g; $MgSO_4.7H_2O$, 0.5 g; Soluble starch, 15 g; Agar, 20.0 g; Distilled water, 750 ml; Tap water, 250 ml) plates supplemented with streptomycin and rose bengal @ 50 mg/L each. In this method, 10 g of compost sample was taken in a 250 ml Erlenmeyer flask containing 90 ml of sterile water and shaken on a rotary shaker for 1 hour. Various dilutions (10^{-2}, 10^{-3}, and 10^{-4}) were used for the isolation of thermophilic fungi. Plates were incubated at 45°C in the dark and were screened daily for up to 5 days. Representative isolates were purified and maintained on YpSs agar slants at 4°C.

Thermophilic fungi tested

Four species of thermophilic fungi namely, *Chaetomium thermophile* La Touch, *Malbranchea sulfurea* (Miehe) Sigler and Carmichael, *Thermomyces lanuginosus* Tsiklinskya and *Torula thermophila* Cooney and Emerson were isolated and selected for growth stimulation of *A. bisporus*. The criterion for selection was their high affinity for cellulose breakdown (Rosenberg, 1978). Though *T. lanuginosus* is a non-cellulolytic fungus yet it was selected as it enhances cellulose breakdown by ce11ulolytic fungi in combination (Deacon, 1985). All the four fungi were tested singly and in different combinations on sterilized compost for their potential to promote *A. bisporus* growth. A control without inoculation was simultaneously run. Culture tubes (160 x 25 mm), each holding a glass tube of 10 mm diameter, were fil1ed with young compost. The glass tube was removed to leave a ventilation channel in the compost (Fig. 1A). The young compost contained in culture tubes was pasteurized for 4 h at 70°C and after stabilizing at room temperature, the compost was inoculated in replicates of three with an 8 mm agar disc of the isolate to be tested singly and

in various combinations (Table 2). The inoculated tubes were placed in polyethylene bags and incubated at 45°C in humidified containers. Growth was recorded daily for the successful1 colonization of the isolate/s. This compost treated with various thermophilic fungi was later used for the inoculation of *A. bisporus* mycelia in test tubes.

Culture of mushroom mycelia in compost in test tubes and Petri dishes

Ten spawn grains covered with *A. bisporus* were put at the bottom of each sterilized culture tube (160 x 25 mm) as an inoculum. Thirty gram substrate (compost treated as above with various thermophilic fungi) was added in the test tubes with sterilized forceps. The culture tubes were closed with sterile cotton plugs and incubated upright at 24±1°C in the dark. All the treatments had three replicates. From first day onwards the position of the mycelial front was recorded with a marker at various intervals. Growth rate was expressed as Kr (mm d^{-1}).

Radial growth rates of *A. bisporus* were also studied in Petri-dishes. Sterile compost and sterile compost fully grown with *T. thermophila* and *M. sulfurea* and a combination of both these fungi (having passed a selection for better growth stimulation) was taken in 90 mm Petri dishes at 30 g per dish. The dishes were inoculated with *A. bisporus* spawn in the center. The lids of the inoculated Petri dishes were fixed with adhesive tape and marked with radii that crossed the inoculum. The dishes were placed in humidified container to prevent desiccation and incubated at 24±1°C in the dark. The position of the mycelial front was marked on third day of incubation and growth was recorded at 2 days interval. Growth rate (GR) was calculated in mm d^{-1}.

Cropping of Agaricus bisporus

Cropping trials for *A. bisporus* were done in polyethylene bags (18 x 12 inches). Young compost (5 kg) was filled in polyethylene bags and pasteurized at 70°C for 8 hr. in an incubator. After pasteurization, the compost bags were stabilized at room temperature and were inoculated with *T. thermophila* and *M. sulfurea* singly and in dual cultures in replicates of five. The bags were incubated at 45°C for 6 days in humidified incubators. Five bags processed similarly but not inoculated with the test organisms served as controls. Side vents in the incubators helped in air circulation. After conditioning of the compost at 45°C, the bags were stabilized at room temperature and inoculated with *A. bisporus* spawn @ 0.5%. After spawning, the compost was pressed hard to make it compact, covered with newspaper sheets sprayed with 2%

formalin and incubated for 15 days at 24±1°C. Mushroom was cultivated and yield was taken as an average of five replications.

Statistical analysis

Within experiments, each treatment performed three to five replicates. Calculations were done with data from independent experiments. Means of n experiments are given. Coefficient of variations (CV) was calculated by analysis of variance (ANOVA) as outlined in Gomez and Gomez (1984). Significant differences were calculated by pair comparison using Duncan Multiple Range Test (DMRT) at P = 0.05.

Results and discussion

Survey

Eighteen species of thermophilic and thermotolerant fungi were isolated from the mushroom compost (Table 1) and these represent most of the known thermophilic taxa. Fast growing species were very common, and after 2 days of incubation counting was possible only if the number of colonies per plate was as small as 15-20. Therefore, higher dilutions (10^{-3}) were used for the isolation of fungi in pure form. During phase-I isolation, it was found that most of the time; the plates were overcrowded with the ubiquitous *Aspergillus fumigatus* and *Rhizomucor* spp. The isolation of a few isolates producing only sterile mycelium proved problematic. Macroscopically, young cultures of *Torula thermophila* and *Chaetomium thermophile* resembled closely and were fast growing. *Rhizomucor pusillus, R. miehei* and *Absidia corymbifera* were also very similar in gross morphology and were fast growing. Seven thermophilic/ thermotolerant fungi were isolated from phase-I compost, rest of the fungi were isolated from phase-II compost (Table 1). The zygomycetous fungi (Table 1) are very common in wheat straw compost (Fergus, 1964; Chang and Hudson, 1967; Chahal *et al*., 1976; Straatsma *et al*., 1994b). During phase-I total count was quite low as revealed by low CFU g^{-1} (Table 1). Fungi isolated from Phase-II compost were *Chaetomium thermophile, Emericella nidulans, Thermoascus aurantiacus, Myriococcum albomyces, Humicola insolens, Malbranchea sulfurea, Torula thermophila, Stilbella thermophila* and *Thermomyces lanuginosus.* Two basidiomycetous species were also visually observed in phase-II compost. A species producing only sterile mycelium was also isolated from phase-II. Most species almost disappeared after phase-II composting. Fungi recovered from end phase-II compost were almost

exclusively *T. thermophila, H. insolens* and *C. thermophile.* The population density of *T thermophila* was the highest (15849 CFU g^{-1}) as compared to other two fungi (Table 1).

The existence of phase-I fungi was limited to the short period before the temperature reached its maximum. With the exception of *T. lanuginosus* they did not reappeared in the phase II compost. The first phase consisted of primary colonizers or primary sugar fungi. In the second phase, the fungi recovered were almost all cellulolytic, suggesting a role in decomposition of organic matter (Rosenberg, 1978; Srivastava *et al.*, 1981) The ratio of thermophilic to mesophilic fungi rose during composting, resulting in a much higher proportion of thermophiles (Chang and Hudson, 1967). The general point that can be made from the survey is that the thermophilic fungi can tolerate the high peak heating phase and their spores persist and remain viable in the compost for a long time. This is suggested by their recolonization of compost after it had cooled down.

Our survey of thermophilic fungi in composts provided us with valuable isolates of *T. thermophila, C. thermophile, M. sulfurea* and *T. lanuginosus.* The first three fungi are known to adapt and colonize the compost frequently (Straatsma *et al*., 1994 a,b) and *T. lanuginosus* is known to promote decomposition rate when grown in combination with cellulolytic fungi (Deacon, 1985).

Growth of thermophilic fungi

Four fungi viz. *Chaetomium thermophile, Malbranchea sulfurea, Thermomyces lanuginosus* and *Torula thermophila* singly and in various combinations were tested on pasteurized compost, and most, in particular *T. thermophila* grew well (Fig. 1A). Of the other species tested, *C. thermophile* and *M. sulfurea* were successfu1 colonizers of compost. *Torula thermophila* grew best in combination with *M. suIfurea* and *C. thermophile* and rapidly colonized the pasteurized compost in test tubes (Fig. 1A). The common compost species *T. lanuginosus* grew poorly when inoculated singly and/or in combination, indicating low competitive abilities. The inoculation of thermophilic fungi showed that compost colonization by selected isolates was successful and that microbial manipulation of phase-II composting is possible.

Table 1. Thermophilous fungi isolated from the mushroom compost phase I and II.

Fungus	Log_{10} CFU g^{-1}	Reference(s)[a]
Zygomycetes		
*Absidia corymbifera**	2.9	2,5,14
*Rhizomucor miehei**	3.1	6,14
*Rhizomucor pusillus**	3.4	2-7,10,11,13,14
Ascomycetes		
Chaetomium thermophile	3.7	2-7,10,13
Emericella nidulans	3.0	2,4,14
*Talaromyces emersonii**	2.9	14
*Talaromyces thermophilus**	2.9	5-7,14
Thermoascus aurantiacus	3.6	3,14
Myriococcum albomyces	3.5	8,13,14
Basidiomycetes		
Basidiomycetes 1		
Basidiomycetes 2		
Hyphomycetes		
*Aspergillus fumigatus**	3.9	2-7,10-14
Humicola insolens	*3.9*	
Malbranchea sulfurea	3.1	5,6,14
Torula thermophila	4.2	2-7,10-14
Stilbella thermophila	2.8	4,7,11,13,14
*Thermomyces lanuginosus**	3.7	2-7,10,11,13,14
Unidentified taxon	2.8	

1. Anonymous (1992), 2. Basuki (1981), 3. Bilai (1984), 4. Renard and Cailleux (1973), 5. Chang and Hudson (1967), 6. Eicker (1977), 7. Fergus (1964), 8. Fergus (1971), 9. Fergus and Sinden (1969), 10. Fermor *et al*. (1979), 11. Hayes (1969), 12. Olivier and Guillaumes (1976), 13. Seal and Eggins (1976), 14 Straatsma *et al*. (1994b)

* Isolated from phase-I compost only, rest of the fungi were isolated from phase II compost

Growth promotion of Agaricus bisporus

The linear growth rate of mycelium of *A. bisporus* on sterilized compost in tubes (Fig. 1B) was 6.1 mm per day. After an initial lag phase of one day, the growth rates were constant (data not shown). The effects of *T. lanuginosus* when inoculated singly or in combination with other thermophilic fungus/fungi on compost were not significant, resulting in lower growth rates as compared to control (Table 2). This could be due to the competition between organisms for producing hydrolytic enzymes. Therefore, it is difficult to establish the initial growth rates of thermophilic fungi. The other three species *viz. C. thermophile, M. sulfurea* and *T. thermophila* tested, promoted growth of *A. bisporus* as the rates of 6.3, 7.1 and 6.6 mm per day respectively when inoculated singly. A mixed inoculum consisting of *M sulfurea* and *T.*

thermophila was the best and promoted growth of *A. bisporus* mycelium to the plateau of 7.7 mm per day (Table 2) and was significantly higher from all other treatments. This finding indicates some specificity of the growth promoting factor(s).

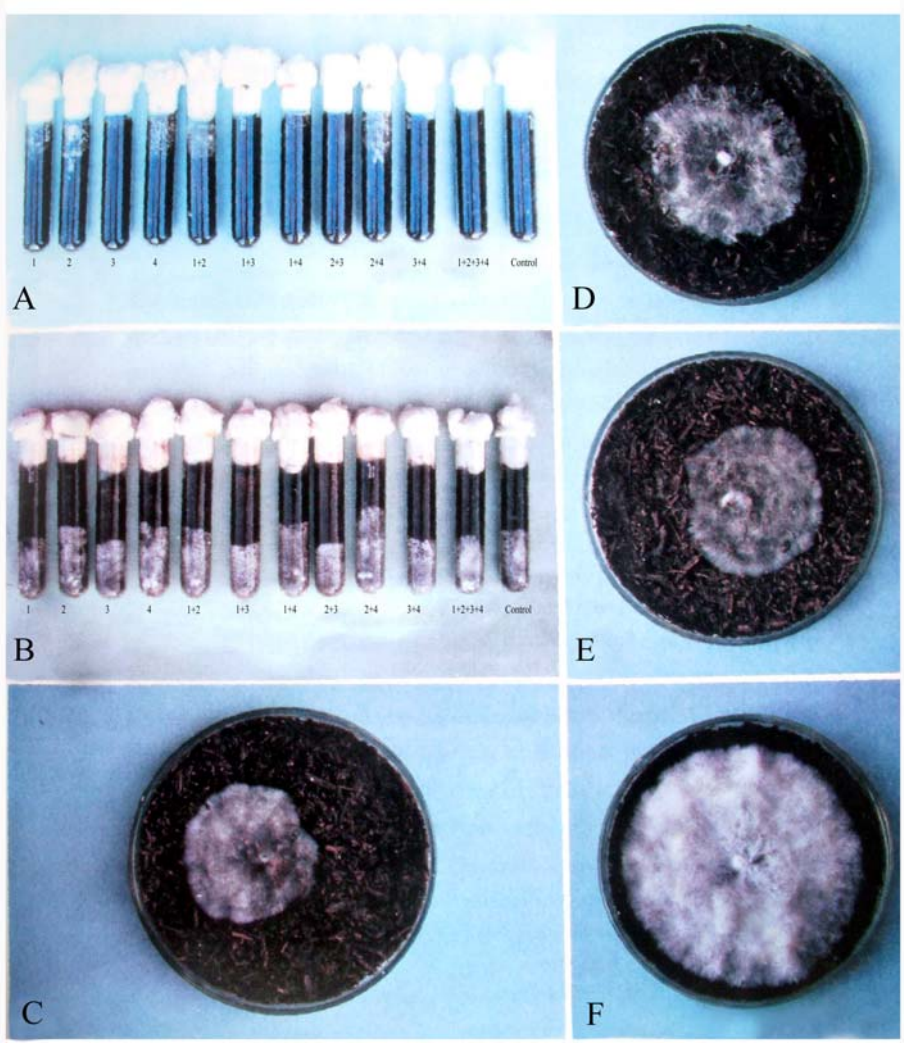

Fig. 1. A. Young compost colonized by four thermophilic fungi, 1. *Chaetomium thermophile*, 2. *Torula thermophila*, 3. *Thermomyces lanuginosus*, and 4. *Malbranchea sulfurea*; B. Colonization of *Agaricus bisporus* mycelium on compost treated with various thermophilic fungi as in A; C-F: Mycelial growth of *Agaricus bisporus* 8 days after inoculation on (C) sterile compost, (D) compost treated with *M. sulfurea*, (E) compost treated with *T. thermophila* and (F) compost treated with *M. sulfurea* + *T. thermophila*.

Table 2. Growth rates of *Agaricus bisporus* on sterilized compost inoculated with different thermophilic fungi singly and in combinations.

Species	Mycelial extension rate (Kr) in tubes (mm/day)[a]	Radial growth rate (GR) in Petri dishes (mm/day)
Control	6.1e	4.9±0.5
Chaetomium thermophile	6.3d	ND
Malbranchea sulfurea	7.1b	6.1±0.9
Thermomyces lanuginosus	5.4g	ND
Torula thermophila	6.6c	5.3±0.8
C. thermophile + M. sulfurea	7.1b	ND
C. thermophile + T. lanuginosus	6.0e	ND
C. thermophile + T. thermophila	6.6c	ND
M. sulfurea + T. lanuginosus	5.8f	ND
M. sulfurea + T. thermophila	7.7a	ND
T. lanuginosus + T. thermophila	6.0e	ND
C. thermophile + M. sulfurea + T. lanuginosus + T. thermophila	6.5c	9.0±1.0

CV = 1.5%, ND = not determined, ± S.D.

[a]Treatments receiving the same letter are not significantly different (DMRT; P<0.05)

Growing *Agaricus bisporus* when grown in Petri dishes on compost inoculated with *M. sulfurea*,(Fig.1D) *T. thermophila* (Fig. 1E) and *M. sulfurea + T. thermophila* (Fig.1 F) showed radial growth rates of (GR) 6.1, 5.3 and 9 mm/day, respectively (Fig.1) The GR on control was low (4.9 mm per day). These two species of thermophilic fungi appeared to be the most promising and were used for more controlled preparation of the substrate for *A. bisporus* cultivation. The growth of *A. bisporus* on control dishes was densed as compared to growth on compost treated with either *M. sulfurea* or *T. thermophila*. Fluffy growth occurred when compost treated with both the fungi that were used as substrate *for A. bisporus* growth (Fig. 1 D, E).

The effect of thermophilic fungi on growth rate of mushroom mycelia in sterilized compost is quite remarkable. Radial growth rate of mushroom mycelia on any laboratory medium never exceeds 3 mm per day (Last *et al*., 1974). Based on our experimental data, we were convinced that thermophilic fungi in particular *T. thermophila* and *M. sulfurea* provides a trigger for enhanced growth of *A. bisporus* acting by an unknown mechanism. This may be taken as an indication that the results of this study could be extrapolated to what actually happens during the production of mushroom compost. Unfortunately a growth-promoting effect of S*cytalidium thermophlium* (syn. = *Torula thermophila*) on *A. bisporus* is not found on agar media (Renard and Cailleux, 1973). Actinomycetes and other bacteria might play a role in successful colonization of *S. thermophilum* during composting (Straatsma *et*

al., 1989). Till (1962) showed that good yield of mushrooms can be obtained on a non-composted sterile mixture containing mainly straw and organic nitrogen. The high hyphal extension rates of *A. bisporus* on compost in the presence of thermophilic fungi may have an ecological significance: it may be able to grow as fast as possible, thereby colonizing as much substrate as possible. Once the substrate has been occupied, the mushroom mycelium seems to be able to prevent the occupation by other microorganisms, either by consuming them (Fermor and Wood, 1981; Fermor and Grant, 1985) or by excretion of carbon monoxide (Stoller, 1978), which effectively inhibits growth of most competing organisms but inhibits the growth of the mushroom mycelium itself only partly (Derikx *et al*., 1990). Carbon dioxide concentrations in the range of 0.3 to 1.0% generate a higher extension rate of mushroom mycelium (Wiegant *et al*., 1992).

The lower growth rates observed in Petri dishes than in culture tubes remain unexplained. This has also been reported for other fungi (Dickson, 1935; Trinci, 1973). The probable reason of higher growth rate in culture tubes may be the ventilation caused by the ventilation channel in tubes.

Table 3. Compost inoculation with thermophilic fungi and cropping of *Agaricus bisporus*.

Treatment	pH of compost Before spawning	Yield of mushrooms g/ 5 Kg of compost)[a]	Biological efficiency (%)
Control	6.9	1020	20.4
M. sulfurea	6.5	1910	38.2
T. thermophila	5.3	1355	27.1
M. sulfurea + *T. thermophila*	6.0	1990	39.8

CV = 2.18%

[a]Average of five replications, the differences among treatments are significant (DMRT; $P<0.05$)

Cropping of Agaricus bisporus

After inoculation with *A. bisporus* spawn at the rate of 0.5% (w/w) and incubation for 15 days, the inoculated composts were fully colonized by *A. bisporus* mycelium. The pH of inoculated compost before spawning was 6.5 and that of the control was 6.9, indicating weaker colonization (Gerrits, 1988b). Mushroom yields from compost inoculated with *M. sulfurea* + *T. thermophila* were high (1990 g/5 kg of compost), almost twice that from pasteurized control. The yield from control compost was clearly lower (1020 g/5 kg of compost) than inoculated compost and was significantly lower as shown by pair comparison using DMRT (Table 3). Low yields of *A. bisporus*

linked to the absence of thermophilic fungi that might be explained by non-selective and/or toxic properties of experimental composts. Composts could have been toxic because of high pH and NH^{+}_{4} values that normally resulted in excessive amounts of volatile ammonia (Gerrits, 1987, 1977).

Inoculation of compost with selective isolates seems important in commercial practice. The advantage gained from pasteurization and inoculation is the early disappearance of NH_3 from compost, which is of interest because growth of *A. bisporus* mycelia requires the absence of NH_3. Another advantage of inoculating thermophilic fungi is the high biological efficiency (39.8%) recovered from the mushroom compost compared to control (20.4%). Respiratory CO_2 of thermophilic fungi plays a stimulatory role (Wiegant, 1992). Our survey of commercial composts showed that *T. thermophila* is naturally presented in compost and might have originated from wheat straw (Chang and Hudson, 1967). Ross and Harris (1983) suspected that a viable but dormant biomass should be presented to fill an otherwise biological vacuum. This prevents colonization by unwanted competitors. Our work focused on thermophiles promoting mycelial growth of *A. bisporus*, and obtaining a good crop on compost precolonized with stimulating organisms may be considered as a way to shorten the process of composting.

The presence of thermophilic fungi is important for successful colonization of *A. bisporus* mycelium. The pre-colonization of mushroom compost by *T. thermophila or M. sulfurea* or a mixture of both these fungi plays a key role in phase-II composting, resulting in a selective substrate. The composting process should thus be optimized by maintaining the temperature to near optimal growth conditions of thermophiles *viz.* 45°C and avoiding the excessive levels of free NH_3.

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