Degradation of Fungal and Actinomycete Mycelia by *Agaricus bisporus*

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(Received 26 November 1984)

Commercial wheat straw/horse manure compost used for production of the cultivated mushroom *Agaricus bisporus* contains a fungal and actinomycete microbial biomass of some 1.5% before inoculation with *A. bisporus*. It has been shown that these mycelia can be used by *A. bisporus* as a sole source of carbon, nitrogen and phosphorus. Mycelium of the thermophilic fungus *Humicola insolens* var. *thermoidea*, a major component of compost microbial biomass, was digested over a 28 d period by *A. bisporus* growing in liquid culture, as determined by light and electron microscopy. Mycelial walls of *H. insolens* var. *thermoidea* contained (by wt) 30% glucose, 22% glucosamine, 6–8% galactose, 2–3% mannose and traces of other sugars. Enzymes that possibly degrade fungal and actinomycete cell walls were produced by *A. bisporus* during growth on such mycelia, and activities of other extracellular enzymes involved in the decomposition of cytoplasmic contents were determined.

INTRODUCTION

The edible mushroom *Agaricus bisporus* is grown commercially on composted horse manure and wheat straw mixtures. During composting the straw surfaces become coated with an amorphous polysaccharide matrix in which are embedded microbial cells and debris (Eddy & Jacobs, 1976; Atkey & Wood, 1983) and by the end of the composting process some 1.5% of the dry weight is fungal and actinomycete mycelia (Sparling *et al.*, 1982). These surface layers on the straw disappear during colonization of the compost by mushroom mycelium, and may thus be a nutrient source for the mushroom. Growth of *A. bisporus* on compost bacteria has been demonstrated (Fermor & Wood, 1981). The present study shows that fungal and actinomycete mycelia can also be used by *Agaricus* as its sole source of carbon, nitrogen and phosphorus.

Grant *et al.* (1984) found that extracellular enzymes produced by *A. bisporus* lysed bacterial cell walls. The composition of the mycelial walls of *Humicola insolens* var. *thermoidea*, a major fungal component of the compost microbial biomass, has been investigated in this work in order to assay for possible wall-degrading enzymes produced by *A. bisporus*. Other extracellular enzymes probably involved in the decomposition of fungal cytoplasmic contents are also described.

METHODS

Organisms. *Humicola insolens* var. *thermoidea* (Cooney & Emerson) comb. nov. (Ellis, 1982) was isolated by direct plating of straws from a commercial horse manure/wheat straw compost. The straw sample was taken immediately before inoculation with *A. bisporus* ('spawning') and isolations were made on malt extract agar (2%, w/v) incubated at 45 °C for 5 d. Four thermophilic actinomycetes that are commonly found in compost (*Streptomyces thermovulgaris*, *Streptomyces* sp. B, *Thermoactinomyces vulgaris* and *Saccharomonospora viridis*) were isolated from a rapidly prepared non-manure, straw compost (Smith, 1983) using half-strength nutrient agar (Oxoid) incubated at 50 °C for 6 d. *Streptomyces clavuligerus* and *Penicillium chrysogenum* were obtained as autoclaved mycelia from commercial antibiotic-producing fermentations (Beecham Pharmaceuticals). *Agaricus bisporus* D621 of commercial origin was used.

Bulk culture of organisms. *H. insolens* var. *thermoidea* inoculum was grown overnight (50 °C, 150 r.p.m.) in two flasks (250 ml) each containing Czapek Dox liquid medium (Oxoid; 100 ml). For batch culture, Czapek Dox liquid medium (20 l) was supplemented with yeast extract powder (Oxoid; 0.5 g l⁻¹) and autoclaved (103 kPa, 40 min). The medium was inoculated with *H. insolens* var. *thermoidea* and stirred, aerated and incubated at 45 °C for 48 h. The fungal pellets were harvested by sieving through 'Tygan' mesh and rinsed with copious quantities of distilled water. Examination by light microscopy showed very few spores were present. The mycelium was then freeze-dried and finely powdered using a pestle and mortar. Mycelium for wall preparations was grown for only 7–8 h to ensure almost total absence of spores.

Actinomycete mycelium was produced in nutrient broth (Oxoid) shaken at 150 r.p.m. for 48 h at 45 °C.

Media and culture of *A. bisporus*. Basal salts medium (Treschow, 1944; see Fermor & Wood, 1981) was used as the standard medium in determining production of transparent 'clearing' zones by *A. bisporus* in opaque solid medium containing suspended, washed and autoclaved fungal or actinomycete mycelia. The pH of these media was maintained by phosphate buffers which were added separately after autoclaving (103 kPa, 20 min); vitamins were added after filter sterilization (Fermor & Wood, 1981). Plates were inoculated with *A. bisporus* D621 (mycelial disc, 6 mm diam.) and incubated at 25 °C.

In the liquid medium used to study the pattern of extracellular enzyme production by *A. bisporus* on fungal mycelium, the phosphate buffer was replaced by 0.05 M-MES buffer pH 6.0 (100 ml l⁻¹), so that *H. insolens* var. *thermoidea* mycelium was the sole source of carbon, nitrogen and phosphorus for mushroom growth. Each flask (250 ml capacity) contained Treschow's basal salts medium (25 ml) plus powdered mycelium of *H. insolens* var. *thermoidea* (250 mg) and was inoculated with *A. bisporus* D621 (0.1 ml; prepared as described by Fermor & Wood, 1981) and incubated at 25 °C. Cultures were gently hand-shaken for 1 min per day during incubation.

Preparation of mycelial walls of *H. insolens* var. *thermoidea*. Mycelium was harvested and washed with ice-cold 1 M-NaCl in 0.1 M-Tris buffer pH 8.0, rather than water, to minimize clumping of walls after hyphal breakage. The mycelium was broken by shaking with Ballotini beads (0.10–0.11 mm diam.) in a Braun Model MSK cell homogenizer, cooled with liquid CO₂. Breakage was monitored by phase-contrast microscopy and was complete after 3 min at 4000 r.p.m. The broken hyphae were washed at least 12 times with the cold NaCl/buffer solution, centrifuging at 5000 *g* after each wash. When all phase-dark material had been removed, the walls were washed three times with distilled water and freeze-dried. Acid hydrolysates of these walls contained only traces of amino acids, indicating that contamination with cytoplasmic material was slight.

Wall fractionation and analysis. Walls were chemically fractionated by the procedure of Mahadevan & Tatum (1965) into fractions I (alkali-soluble), II (acid-soluble), III (acid-insoluble, alkali-soluble) and IV (acid- and alkali-insoluble). These fractions and whole walls were analysed qualitatively by descending paper chromatography of acid hydrolysates on Whatman no. 1 or no. 3 MM paper in the following solvents: A, butan-1-ol/pyridine/glacial acetic acid/water (60:40:3:30, by vol.) (for hexosamines and amino acids); and B, ethyl acetate/pyridine/water (8:2:1, by vol.) (for neutral sugars). Neutral sugars were detected with alkaline AgNO₃ (Trevelyan *et al.*, 1950); hexosamines and amino acids with ninhydrin (0.2% in acetone); and uronic acids with aniline-diphenylamine reagent (Dawson *et al.*, 1969). For analysis of neutral sugars, walls were hydrolysed under N₂ in 11.25 M-H₂SO₄ at 30 °C for 3 h, followed by dilution to 0.43 M-H₂SO₄ and heating at 96–98 °C for 4 h, again under N₂ (Bartnicki-Garcia, 1966). Acid was neutralized with BaCO₃. For glucosamine analysis, hydrolysis was in 6 M-HCl at 100 °C for 4 h or 6 h under N₂. Deacidification was by repeated evaporation to dryness over P₂O₅ and NaOH pellets.

Glucose was determined with glucose oxidase (TEKIT 952 DM; Searle Diagnostics Inc., Chicago, USA), galactose with galactose oxidase (Roth *et al.*, 1965), and mannose by elution of the mannose regions from paper chromatograms of hydrolysates, followed by measurement of reducing sugar (Lever, 1972). Standards and blanks were chromatographed on and eluted from the same paper. Glucosamine was determined by quantitative paper chromatography of hydrolysates and standards in solvent A, followed by staining the dried papers with the cadmium acetate/ninhydrin mixture of Heilmann *et al.* (1957), elution of the spots with methanol, and colorimetry at 500 nm. The results obtained were confirmed by hexosamine analysis by the Elson–Morgan procedure of Gatt & Berman (1966).

The action of laminarinase (mollusc; Sigma) on fraction III was assayed in 0.05 M-sodium citrate buffer pH 5.0 at 37 °C, by measuring glucose released. The action of chitinase (*Streptomyces*; Sigma) on fraction IV was assayed after thoroughly suspending the fraction in 0.05 M-sodium citrate buffer pH 6.0, and measuring release of reducing sugar. The *N*-acetylglucosamine released was identified by co-chromatography with an authentic sample in solvent A.

IR spectra of fraction IV and authentic chitin (crustacean shells; Sigma) were obtained on a Unicam SP-200 spectrophotometer after powdering the samples with KCl and pressing into discs.

Enzyme assays. Centrifuged culture supernatants were assayed for extracellular enzyme activity using the techniques described by Fermor & Wood (1981). All assays were done on at least three replicates. β -*N*-Acetylgalactosaminidase (EC 3.2.1.53) and β -*N*-acetylglucosaminidase (EC 3.2.1.30) were assayed spectrophotometrically using the corresponding *p*-nitrophenyl substrates (Sigma). β -*N*-Acetylmuramidase (lysozyme) (EC

3.2.1.17) was assayed by measuring the decrease in optical density of a suspension of *Micrococcus lysodeikticus* (Sigma) at 540 nm in a Pye Unicam SP 30 spectrophotometer. The chitinolytic activity of supernatants was assessed by adding supernatant (1.0 ml) to a suspension of purified chitin (Sigma; 10 mg ml⁻¹) in 0.05 M-citrate/phosphate buffer pH 6.6 (1.0 ml) and incubating it at 25 °C for 1 h (Monreal & Reese, 1969). The reducing sugar released by chitinase activity (EC 3.2.1.14) was measured by the Nelson–Somogyi method using a standard curve for *N*-acetylglucosamine read at 500 nm. Before assay for nucleases, supernatants were concentrated 15- to 20-fold by ultrafiltration over a PM10 membrane (Amicon) and then an agar diffusion technique was used (Fermor & Wood, 1981). Laccase (EC 1.10.3.2) was assayed polarographically and was used as a growth marker for *A. bisporus* (Wood & Goodenough, 1977). To assay laminarinase activity (EC 3.2.1.6), culture supernatant (0.25 ml) was added to laminarin substrate (Calbiochem; 20 mg ml⁻¹ in 0.25 ml distilled water) in 0.05 M-acetate buffer pH 5.0 (0.5 ml), and incubated for 1 h at 25 °C. The reducing sugars released were measured by the Nelson–Somogyi method using a standard curve for D-glucose read at 500 nm. Lipase (EC 3.1.1.3) was assayed using a modified agar diffusion method with tributyrin as substrate. Acid, neutral and alkaline proteases were measured colorimetrically by liberation of dye from an insoluble hide powder substrate conjugated with Remazol Brilliant Blue (Rinderknecht *et al.*, 1968).

RESULTS

Growth of A. bisporus on actinomycete and fungal mycelium in agar

A. bisporus produced transparent zones in the opaque Treschow's basal salts medium (cleared zones) when the medium contained washed, autoclaved mycelial suspensions of the actinomycetes *Streptomyces clavuligerus*, *Streptomyces thermovulgaris*, *Streptomyces* sp. B, *Saccharomonospora viridis* and *Thermoactinomyces vulgaris* as sole carbon and nitrogen sources. Sections of agar gel taken from 'cleared zones' were stained and examined by light microscopy. They showed an almost total absence of actinomycete mycelium (0.5–0.75 µm diam.) which was easily distinguished from *A. bisporus* mycelium (2.5–5.0 µm diam.) by its smaller hyphal diameter. *A. bisporus* mycelial growth on media containing the three *Streptomyces* species could not be accurately measured as it was sparse and did not form a coherent mycelial front. The *A. bisporus* hyphae growing on these actinomycetes were highly branched in comparison with those hyphae growing on malt extract agar (2%, w/v). In contrast, *A. bisporus* mycelium growing on *T. vulgaris* and *Sacch. viridis* was sparse but grew in thick infrequently branched strands.

A. bisporus also produced 'cleared zones' in opaque media containing either *H. insolens* var. *thermoidea* or *P. chrysogenum* fungal mycelium. The colony radial extension rate of *A. bisporus* mycelium on these media was about 1.0 mm d⁻¹. Examination by light microscopy of the 'cleared zones' revealed an almost total absence of non-Basidiomycete mycelium. The distance of 'cleared zones' in advance of the growing mycelial front (on media containing suspended *Humicola* or *Penicillium* mycelium) could be increased by incubating the plates in a refrigerator, which slowed hyphal growth whilst allowing extracellular enzymes to diffuse through the media.

Composition of mycelial walls of H. insolens var. thermoidea

The walls contained (by wt) 30% glucose, 22% glucosamine, 6–8% galactose, 2–3% mannose and traces of other sugars. Galactosamine and uronic acids were not present. Chemical fractionation of the walls yielded two main polysaccharide-containing fractions. Fraction III formed a gel with water, accounted for 15% of the original wall weight, and on acid hydrolysis produced only glucose, with small amounts of oligosaccharides. Treatment with laminarinase resulted in the release of 50% of the glucose residues as free glucose. Fraction IV was insoluble in water and accounted for 23% of the original wall. Acid hydrolysis converted this fraction to free glucosamine with traces of glucose. Treatment with chitinase released only small amounts of *N*-acetylglucosamine (approx. 5% of total glucosamine present) in solution. However, the IR spectrum of this fraction was virtually indistinguishable from that of an authentic chitin sample. Fraction I from the Mahadevan & Tatum (1965) procedure contained 10% of the wall weight as poly- or oligosaccharide(s) of glucose, galactose and mannose (approx. ratio 6:3:1). Fraction II accounted for almost 50% of the wall weight as free glucose, galactose and mannose (approx. ratio 3:1:1) and traces of glucosamine, these being acid hydrolysis products of the polysaccharides present in the other fractions.

Fig. 1. *Agaricus bisporus* (A) growing on mycelium of *Humicola insolens* var. *thermoidea* (H) as sole carbon, nitrogen and phosphorus sources in liquid culture (14 d after inoculation). Magnification $\times 400$.

Table 1. Enzyme activities produced by *A. bisporus* growing on mycelium of *H. insolens* var. *thermoidea*

Enzyme	Possible substrate	Time (d) . . . 0	7	14	21	28	56*
		pH of supernatant . . . 5.5	5.4	5.6	5.7	5.8	5.7
β -N-Acetylgalactos-aminidase†	Cell wall	ND	ND	ND	ND	ND	0.19
DNAase	DNA	—	—	—	—	—	+
Laccase‡	(Growth marker)	ND	0.010	0.031	0.120	0.084	0.199
Laminarinase (endo-1,3- β -D-glucanase)§	Cell wall glucans	—	ND	ND	ND	22.0	19.0
Lipase	Lipids	—	ND	ND	+	+	+
Protease	Protein	—	ND	ND	2.11	3.33	10.70
Acid pH 3.6		—	ND	ND	7.16	9.11	25.68
Neutral pH 7.0		—	ND	ND	6.79	7.18	24.02
Alkaline pH 9.1		—	ND	ND	—	—	—
RNAase	RNA	—	—	—	—	—	+

—, Not determined; ND, not detected; +, enzyme present.

* All *Humicola* mycelium degraded, as observed by light microscopy.

† Expressed as μmol nitrophenol released (ml supernatant) $^{-1}$ min $^{-1}$.

‡ Expressed as μmol O₂ released (ml supernatant) $^{-1}$ min $^{-1}$.

§ Expressed as μg reducing sugar (ml supernatant) $^{-1}$ min $^{-1}$.

|| Expressed as μg Remazol Brilliant Blue solubilized (ml supernatant) $^{-1}$ h $^{-1}$.

Growth of A. bisporus on mycelium of H. insolens var. thermoidea in liquid medium

After an early lag phase *A. bisporus* grew on mycelium of *H. insolens* var. *thermoidea* as sole carbon, nitrogen and phosphorus sources, as shown by light microscopy and by the increase in activity of the enzyme laccase which is proportional to mycelial dry weight (Wood, 1979) (Fig. 1, Table 1). Degradation of mycelium of *H. insolens* var. *thermoidea* (Fig. 1) was confirmed by electron microscopy. Culture supernatants were assayed for extracellular enzymes (Table 1), which could account for the degradation of fungal wall polymers and intracellular lipids, nucleic acids and proteins. Highest enzyme activities were recorded after 21 d and by 28 d no visible mycelium of *H. insolens* var. *thermoidea* remained in the cultures. Activities of β -N-acetylglucosaminidase and β -N-acetylmuramidase, which were induced in the presence of bacterial cell walls (Fermor & Wood, 1981; Grant *et al.*, 1984), were not detected in the presence of mycelium of *H. insolens* var. *thermoidea*. Moreover, chitinase activity was not detected by the assay techniques used. Nucleases were only detected after the supernatants were concentrated 15- to 20-fold. The pH of the culture supernatants remained constant throughout the 56 d duration of the experiments (Table 1).

DISCUSSION

Fungal and actinomycete mycelia represent a considerable potential nutritional resource for *Agaricus bisporus* growing in compost. The total microbial biomass in a horse manure and wheat straw compost has been estimated immediately before inoculation (spawning), by both direct counts and biochemical methods, as 9.2 mg C (g dry wt compost)⁻¹ (Sparling *et al.*, 1982); this represents approx. 2% of compost dry weight assuming that microbial biomass contains 50% carbon. The ratio of fungal and actinomycete to bacterial biomass in this compost was 2.8:1. However, the relative proportions of fungal to actinomycete mycelia in compost is unknown.

The maximum contribution of the microbial biomass to mushroom biomass has been calculated as less than 10% (Sparling *et al.*, 1982). This suggests that *A. bisporus* probably obtains the bulk of its carbon nutrition from straw. However, the nitrogen content of microbial biomass is high, and since micro-organisms will also concentrate minerals during composting, the biomass could act as a concentrated source of nitrogen and minerals. *Streptomyces clavuligerus* mycelium, for example, contained on average 11% nitrogen by dry weight and *Penicillium chrysogenum* contained 3.4% nitrogen (Jenkinson, 1981), although the mineral content of organisms will vary depending on growth conditions (Van Veen & Paul, 1979).

Compost is cooled to 25 °C before inoculation with mushroom mycelium and so a large proportion of the thermophilic microflora will be near or below its minimum growth temperature. The thermophilic actinomycetes *Saccharomonospora viridis*, *Streptomyces thermo-vulgaris* and *Thermoactinomyces vulgaris* all have a minimum temperature for growth of 27 °C; they will be in a static state at 25 °C, with the mycelial structure remaining intact assuming no autolysis occurs. A significant fraction of the nutrient content of the compost may thus be present within the microflora and available only to micro-organisms which possess the enzyme systems needed to degrade them. Atkey & Wood (1983) observed that actinomycete hyphae, often showing considerable cellular breakdown, were frequently found in close association with *A. bisporus* hyphae, particularly towards the end of cropping. *Humicola insolens* var. *thermoidea* has a minimum temperature for growth of 23 °C (Cooney & Emerson, 1964) and yet is isolated only occasionally from spent compost at the end of cropping.

The actinomycetes tested represent three of the four major actinomycete cell wall types as recognized by Gottlieb (1974). All contain *N*-acetylmuramyl- β -1,4-*N*-acetylglucosamine linkages in their peptidoglycans, and it is therefore possible that the β -*N*-acetylmuramidase secreted by *A. bisporus* which degrades walls of *Bacillus subtilis* (Grant *et al.*, 1984) would also be capable of attacking these actinomycete walls.

Investigation of the wall composition of mycelium of *H. insolens* var. *thermoidea* has shown that it is probably of the common chitin-glucan type V (Bartnicki-Garcia, 1968). The glucan contains β (1 $\rightarrow$ 3) linkages, since it was extensively degraded by commercial laminarinase. Laminarinase activity was detected in supernatants of *A. bisporus* growing on *H. insolens* var. *thermoidea* (Table 1). The failure to detect chitinase activity in such supernatants could be explained if the enzyme was produced but was adsorbed on to the insoluble mycelial substrate. This type of enzyme adsorption phenomenon was noted by Manning & Wood (1983) who found that the extracellular endocellulase activity of *A. bisporus* in culture filtrates was inversely related to cellulose concentration in the culture and that free cellulase could be bound to cellulose. Similarly, Grant *et al.* (1984) showed that the extracellular β -*N*-acetylmuramidase activity of *A. bisporus* cultures was considerably higher in cultures containing a lower concentration of bacterial cell walls.

The carbon chain lengths of fatty acids in compost resemble those often found in the hyphae and spores of thermophilic fungi. Micro-organisms, particularly fungi, are probably the only source of polyunsaturated fats in compost and these are important nutrients for *A. bisporus* (Wain, 1981). Although the lipid component of *H. insolens* var. *thermoidea* was not analysed in the present study, lipase activity of *A. bisporus* was measurable after 21 d growth on *Humicola* mycelium.

Attempts to improve compost productivity by inoculation of thermophilic fungi into mushroom compost after completion of composting has had little beneficial effect, as an adequate natural fungal population is already present (Ross & Harris, 1983). Increased

knowledge of the nutritional requirements of the mushroom should result in advances in the ability to manipulate *A. bisporus* for crop production. It is conceivable that a formulated supplement of microbial origin could act as a slowly released nutrient to increase mushroom yield during the later stages of cropping.

We thank Dr I. Normansell, Beecham Pharmaceuticals, Worthing, UK, for providing organisms. We also thank Dr D. Wood for criticism and encouragement, and Sarah Molyneux and Barbara Prosser for skilful technical assistance.

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