Degradation of Bacteria by *Agaricus bisporus* and Other Fungi

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Agaricus bisporus and other fungi (Basidiomycetes, Ascomycetes, Fungi Imperfecti and Phycomycetes) grew and produced transparent zones in opaque media containing killed *Bacillus subtilis* as sole carbon and nitrogen source. Other Gram-positive and Gram-negative bacteria were also degraded by *A. bisporus*. Degradation of *B. subtilis* by *A. bisporus* was examined by light and electron microscopy. Liquid cultures were assayed for extracellular fungal enzymes which included β -N-acetylglucosaminidase, lipase, nucleases and acid, neutral and alkaline proteases.

Microbial biomass in compost may serve as a source of nitrogen, carbon and minerals, and as a water reserve for the growth of *A. bisporus*.

INTRODUCTION

The substrate for growth and fruiting of *Agaricus bisporus* in commerce is prepared by the composting of wheat straw. During this process a defined microbial succession occurs and large numbers of micro-organisms are produced, as shown by plate counts (Fermor *et al.*, 1979; Fermor & Wood, 1979). This accumulated microbial biomass could serve as a major source of nitrogen for the mycelium of the fungus. Micro-organisms could serve also as a source of carbon compounds and a mineral source, as well as a water reserve for the growing mycelium. Recent studies following the recognition of the role of organisms in carbon, nitrogen and phosphorus flows in nature have stressed the role of micro-organisms as sources and sinks for nutrients, in addition to their traditional role in the transformation of nutrient elements (Paul & Voroney, 1980). Many classes of organisms possess bacteriolytic enzymes (Stolp & Starr, 1965; Strominger & Ghuysen, 1967; Gooday, 1979) some of which have been shown to have a nutritional role.

This present investigation had two aims; firstly, to study whether degradation of dead bacteria was a possible mode of nutrition for filamentous fungi, and in particular whether it was used by *A. bisporus*, and secondly, to determine the nature of the extracellular enzymes produced by *A. bisporus* which might be used to degrade bacteria. *Bacillus subtilis* was chosen as the main subject of these experiments because it was the bacterium most frequently isolated from mushroom composts and is also very common in soil.

METHODS

Organisms. *Agaricus bisporus* D621 was used. This is a heterokaryotic fertile strain of commercial origin. Basidiomycete cultures were obtained from the Glasshouse Crops Research Institute collection of fungi. *Phycomyces blakesleeanus*, *Sordaria fimicola* and *Neurospora tetrasperma* were the gift of Dr C. Thurston, Queen Elizabeth College, London. *Bacillus subtilis* 168 derivative S2861 (trivial name 261) *spoIIA69 spoG55 rif-2* (asporogenous) was the gift of Dr P. J. Piggot, National Institute for Medical Research, London. Other bacteria were obtained from the National Collection of Type Cultures, London. All other organisms used in the present study were isolated directly from mushroom compost.

Fungal media and culture. Basal salts medium (Treschow, 1944) was used as the standard medium in all experiments. The composition of this medium is: KCl, 0.2 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g; CaCl_2 , 0.2 g; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 10 mg; $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ (stock solution 15 g l⁻¹), 75 ml; KH_2PO_4 (stock solution 9 g l⁻¹), 15 ml; vitamin solution (20 mg thiamin.HCl and 2 mg biotin in 100 ml distilled water), 1 ml; H_3BO_3 , 0.3 mg; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.25 mg; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 2 mg; $\text{Na}_2\text{MoO}_4 \cdot 7\text{H}_2\text{O}$, 0.4 mg; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 mg; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.7 mg; distilled water to 1 litre. The stock solutions were each sterilized and added to the medium after autoclaving.

For determining growth of *A. bisporus* on a range of Gram-positive and Gram-negative bacteria, bacteria were grown as shake cultures (600 ml) in nutrient broth (Oxoid) and harvested at the end of the exponential phase of growth. Bacterial cultures were centrifuged (15 000 g, 10 min) then rinsed in distilled water. These cells were added to 200 ml medium which contained either Eggins & Pugh salts + yeast extract (Eggins & Pugh, 1962) or basal salts (Treschow, 1944), and 3 g agar (Difco Bacto), and autoclaved (103 kPa, 20 min). Standard volume (20 ml) plates were poured, inoculated with mycelial discs (4 mm diam.) of *A. bisporus* D621 and incubated at 25 °C.

Degradation of *B. subtilis* by filamentous fungi was indicated by the production of transparent zones in the opaque Treschow basal salts medium containing suspended bacteria. The medium was prepared in individual bottles each containing: Treschow basal salts, 20 ml; Ion agar no. 2 (Oxoid), 500 mg; freeze-dried *B. subtilis*, 400 mg. The following were added separately to each bottle after autoclaving (103 kPa, 20 min) from sterile stock solutions: $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ (15 g l⁻¹), 4.9 ml; KH_2PO_4 (9 g l⁻¹), 0.1 ml; vitamin solution (20 mg thiamin.HCl and 2 mg biotin in 100 ml distilled water), 0.1 ml. When the medium (pH 5.7) had solidified, it was inoculated with mycelial discs (4 mm diam.) of filamentous fungi and incubated at 25 °C.

Medium of similar composition, without agar, but with 500 mg bacteria was used in static liquid cultures (25 ml medium per 250 ml flask) to study extracellular enzyme production and dry weight loss and to provide samples for electron microscopy. Each flask of medium was inoculated with a vegetative mycelial suspension of *A. bisporus* (0.1 ml) and incubated at 25 °C. The inocula were prepared as follows. The fungus was grown in three flasks (250 ml capacity with 25 ml liquid malt extract at 20 g l⁻¹) for 4 weeks at 25 °C. Cultures were then centrifuged (10 000 g, 30 min), the supernatant was decanted, the mycelium was rinsed and then added to sterile distilled water (100 ml). The mycelial suspension was shaken with approximately 100 glass beads (5 mm diam.; 400 rev. min⁻¹, 30 min).

Bulk culture of bacteria. For determination of fungal growth (other than *A. bisporus*) on solid media, and for determination of *A. bisporus* growth in liquid media, large-scale cultures of *B. subtilis* 168 (asporogenous) were grown. For this, the bacteria were first subcultured in nutrient broth (Oxoid) followed by growth on nutrient agar at 30 °C. Cells then grown in shaken culture in Spizizen salts medium (S.S.M.; Piggot, 1975) to check culture purity and growth characteristics. S.S.M. contained (in 900 ml): $(\text{NH}_4)_2\text{SO}_4$, 2 g; K_2HPO_4 , 14 g; KH_2PO_4 , 6 g; trisodium citrate. $2\text{H}_2\text{O}$, 1 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g. The final medium consisted of: S.S.M., 900 ml; monosodium glutamate (15 g l⁻¹, adjusted to pH 7.0 with NaOH), 90 ml; glucose (200 g l⁻¹), 10 ml. The components were sterilized separately by autoclaving. All chemicals used were laboratory grade (BDH). These small shake-flask cultures were used to inoculate 20 l fermenter cultures which were, in turn, used to seed four 400 l cultures in S.S.M. The cultures were incubated at 30 °C, and harvested at the end of the exponential phase of growth. The harvested cells were treated as follows: (i) suspended in trichloroacetic acid (100 g l⁻¹) for 2 h at room temperature; (ii) centrifuged (5000 g, 20 min) to remove most of the trichloroacetic acid; (iii) washed three times in copious distilled water to about pH 4.0 (continuous centrifuging M.S.E. 18 at 15 000 g); (iv) extracted with an equal volume of ether, twice, to remove soluble cell contents and residual trichloroacetic acid; (v) freeze-dried.

Enzyme assays. Assays were performed on liquid culture supernatants obtained after centrifugation (18 000 g, 30 min). Supernatants were stored at -20 °C until required; freezing and thawing did not affect enzyme activity. Laccase was assayed polarographically (Wood & Goodenough, 1977). Tyrosinase was assayed using a method similar to that for laccase but substituting 3,4-dihydroxyphenylalanine as substrate. One unit of laccase or tyrosinase was defined as that amount of enzyme catalysing the consumption of 1 $\mu\text{mol O}_2$ min⁻¹. Acid, neutral and alkaline proteases were measured colorimetrically by the liberation of dye from an insoluble substrate, Remazol Brilliant Blue-hide powder conjugate (Rinderknecht *et al.*, 1968). The incubation mixture, which contained 12 mg substrate, 0.6 ml supernatant and 1.2 ml buffer, was incubated at 25 °C and the reaction was terminated by the addition of trichloroacetic acid (500 g l⁻¹). After centrifugation (10 000 g, 10 min) the absorbance was measured spectrophotometrically at 595 nm. Activity was calculated as μg Remazol Brilliant Blue (RBB) solubilized h⁻¹ (ml extract)⁻¹ at 25 °C. β -N-Acetylglucosaminidase assay with a *p*-nitrophenyl glycoside as substrate was carried out as follows. Culture supernatant (0.2 ml) was added to a solution (0.4 ml) of *p*-nitrophenyl-N-acetyl- β -D-glucosaminide (Sigma, 1 mg ml⁻¹) in 0.05 M-acetate buffer (pH 5.0) and incubated at 25 °C for 1 h. The reaction was terminated by the addition of 1 M- Na_2CO_3 (2 ml); the released *p*-nitrophenol was measured spectrophotometrically at 400 nm. Activity was calculated as $\mu\text{mol p-nitrophenol released min}^{-1}$ (ml culture supernatant)⁻¹. Lysozyme was assayed by measuring the decrease in turbidity of a suspension of *Micrococcus lysodeikticus* (Sigma) at 540 nm (Demetriou *et al.*, 1974).

The medium described by Sierra (1957) was used in a preliminary experiment to detect whether *A. bisporus* produced lipase in solid medium; sorbitan monolaurate (Tween 20) was used as the lipid substrate. Later, the

lipase activity of liquid culture supernatant was assayed using a modification of an agar diffusion method (Lawrence *et al.*, 1967). An emulsion of tributyrin (0.5 ml, 10 ml l⁻¹) in water (prepared by sonication, 1.5 A for 5 min) was added to 4.5 ml of a hot solution of agar (Oxoid Ion agar no. 2, 11.1 g l⁻¹ in 0.05 M-phosphate buffer, pH 7.0). This emulsion (5 ml) was spread evenly over a microscope slide (3.75 × 7.5 cm), four holes (6 mm diam.) were made with a steel cutter and 20 µl supernatant was added to each. The slide was placed in a humid chamber and incubated at 25 °C for 65 h, prior to measuring the diameter of cleared zones formed in the opaque medium.

The agar diffusion technique (Lawrence *et al.*, 1967) was used to detect DNAase and RNAase. DNA (calf thymus, Sigma; 1 mg ml⁻¹) or yeast RNA (BDH; 2 mg ml⁻¹) was suspended in the agar medium in 0.1 M-acetate buffer (pH 5.0). To each well was added 20 µl culture supernatant, the slides were placed in humid chambers and incubated at 25 °C. After 2 to 3 d, slides were flooded with 1 M-HCl and clear zones in the opaque medium indicated areas of nuclease activity. As nuclease activity was found to be very low, supernatants were concentrated (15- to 20-fold) by ultrafiltration over a PM10 membrane (Amicon). Supernatants for nuclease assay were taken from cultures in which the phosphate buffer had been replaced by 0.1 M-Tris (the initial pH of the medium was 5.5), as no nuclease activity could be detected in supernatants of cultures in the standard media (on which all the other enzyme assays were performed).

Electron microscopy. Samples of bacteria and bacteria/fungal mycelial mats were taken from liquid cultures and fixed in Karnovsky's fixative for 2 h (Karnovsky, 1965), followed by four 30 min washes in 0.2 M-cacodylate buffer (pH 7.4). Post-fixation in OsO₄ (20 g l⁻¹) was followed by a further four washes in cacodylate buffer. Dehydration by passage through graded water/ethanol mixtures was followed by embedding in Spurr's resin (Spurr, 1969). Sections were post-stained with uranyl acetate (20 g l⁻¹) for 1 h and with lead citrate for 30 min (Reynolds, 1963) prior to examination.

RESULTS

Growth of A. bisporus on bacteria

Agaricus bisporus produced transparent zones in the opaque medium (cleared zones) when grown on media containing autoclaved suspensions of Gram-positive or Gram-negative bacteria (Table 1). The distance of the cleared zones in advance of the growing mycelial front could be increased by incubating the plates in a refrigerator, which slowed hyphal growth whilst allowing extracellular enzymes to diffuse through the media. Sections of agar gel taken from cleared and non-cleared areas were stained and examined by light microscopy. These showed the absence of bacteria in the cleared area (Fig. 1). Colony radial growth rates of *A. bisporus* were 30–60% slower on media containing suspended bacteria than on malt extract agar (20 g l⁻¹), but the hyphae grew more closely. Growth rates of *A. bisporus* were slightly greater on Eggins & Pugh medium (containing carbon and nitrogen sources) than on Treschow basal salts (containing autoclaved bacteria as the sole source of carbon and nitrogen) when both media contained the same species of autoclaved bacteria in suspension.

Growth of fungi on B. subtilis

The majority of the filamentous fungi successfully colonized and degraded *B. subtilis* in solid medium (Table 2). None of the *Agaricus* spp. nor other Basidiomycetes formed fruit-body primordia on this medium in which the bacteria were the sole source of carbon and nitrogen. The ability to utilize dead *B. subtilis* was not restricted to the Basidiomycetes; representatives from the Ascomycetes, Fungi Imperfecti and Phycomycetes were also able to use this mode of nutrition (Table 2). Basidiomycetes grew at approximately half of their normal growth rate on a standard fungal medium, whereas the other classes of fungi grew 5- to 10-fold slower. The morphology of the Fungi Imperfecti was atypical of that normally found on standard fungal media: *Aspergillus fumigatus* sporulated, but the colony colour was grey; *Penicillium* sp., normally blue-green on malt extract agar, grew as a non-sporulating white mycelial colony; *Trichoderma viride*, normally a fast-growing green colony, sporulated but formed an extremely slow-growing velvety-beige colony having distorted hyphae.

Table 1. *Growth of Agaricus bisporus on autoclaved bacteria suspended in solid media*

Bacterial species	Gram reaction	Medium†	Colony radial extension rate‡ (mm d ⁻¹)	Cleared zone
<i>Acinetobacter calcoaceticus</i> NCTC 7844	—	EP	1.14	+
<i>Acinetobacter calcoaceticus</i> NCTC 7844	—	TBS	1.01	+
<i>Alcaligenes faecalis</i> NCTC 655	—	TBS	0.90	+
<i>Bacillus subtilis</i> *	+	EP	0.80	+
<i>Bacillus subtilis</i> 168, wild type	+	EP	0.76	+
<i>Bacillus subtilis</i> 168, asporogenous	+	TBS	0.91	+
<i>Bacillus coagulans</i> *	+	EP	0.61	+
<i>Bacillus stearothermophilus</i> *	+	EP	0.98	+
<i>Bacillus stearothermophilus</i> *	+	TBS	0.91	+
<i>Klebsiella pneumoniae</i> NCTC 418	—	EP	0.92	+
<i>Klebsiella pneumoniae</i> NCTC 418	—	TBS	0.84	+
<i>Mycobacterium phlei</i> NCTC 8151	var.	EP	0.68	+
<i>Proteus vulgaris</i> NCTC 4175	—	EP	ND	+
<i>Staphylococcus epidermidis</i> NCTC 4276	+	EP	ND	—
(<i>Micrococcus</i> sp. subgroup 1)				
<i>Staphylococcus epidermidis</i> NCTC 4276	+	TBS	ND	—
(<i>Micrococcus</i> sp. subgroup 1)				
<i>Escherichia coli</i> NCTC 9001	—	EP	ND	+
Control		ME	1.55	—
Control		EP(G)	1.05	—
Control		TBS	NG	—

* Isolated from compost.

† EP, Eggins & Pugh; TBS, Treschow basal salts; ME, malt extract; EP(G), Eggins & Pugh with 10 g glucose l⁻¹.

‡ ND, Not determined; NG, no growth.

Liquid cultures and electron microscopy

Bacillus subtilis cells were harvested in the late-exponential phase of growth. The fixation and staining procedures used to examine them were shown not to cause cell wall degradation (Fig. 2*a*), though there was a small number (1–2%) of lysed cells in these preparations. However, the cells were distorted by freeze drying (Fig. 2*b*) and needed a period of rehydration before most of them resumed normal cell shapes. Mycelium of *A. bisporus* degraded these *B. subtilis* cells (when used as sole carbon and nitrogen source) in liquid media (Fig. 2*c, d*). Examination with the microscope (after 35 d) revealed several changes in the bacteria from such cultures: bacterial counts were smaller; bacteria were less clumped; cell contents had considerably shrunk or disappeared; cell walls were perforated and wall fragments were apparent; wall thickness was substantially reduced in some bacteria.

Increase in laccase activity, used to estimate *A. bisporus* mycelial growth, was found roughly to correspond with the total dry weight loss in the cultures (Fig. 3), and both followed the pattern expected for *A. bisporus* growth in liquid culture. The pH of the medium showed a large shift from acid to alkaline. Between 14 d after inoculation and the end of the experiments the cultures progressively changed from colourless to dark brown.

Liquid cultures were assayed for the pattern of extracellular enzyme activities (Table 3). β -*N*-Acetylglucosaminidase activity was found (Fig. 4*a*). The enzyme was active over a wide range from pH 2.8 to 8.0. β -*N*-Acetylglucosaminidase activity appeared to be induced by the presence of the bacteria in the liquid cultures. No enzyme activity was detected when *A. bisporus* mycelium was grown in Treschow basal salts medium with glucose and glutamate as the carbon and nitrogen sources instead of bacteria.

Acid, neutral and alkaline proteases were produced by *A. bisporus* (Fig. 4*a*). Neutral protease showed the greatest activity reaching a peak between 20 and 40 d. In addition, *A. bisporus* was grown on an insoluble protein source, casein, in Treschow basal salts medium to

Fig. 1. Growth of *A. bisporus* on Treschow basal salts agar containing autoclaved Gram-negative bacteria (*Alcaligenes faecalis*) as sole carbon and nitrogen source. (a) Well-defined cleared zone formed in advance of the colonizing mycelium. (b) Uncolonized agar with autoclaved bacteria. (c) Colonized agar (from cleared zone) showing very few bacteria present, thus indicating that the majority of the bacteria originally present had been degraded by extracellular enzymes from *A. bisporus* mycelium.

compare the pattern of extracellular proteases produced. Insoluble casein was degraded faster than bacteria; protease activities were 2- to 3-fold higher. Protease production reached a peak after 15 d and both neutral and alkaline proteases had high activity (Fig. 4b).

The formation of lipolytic enzymes by a colony of *A. bisporus* was indicated by a whitish cream precipitate in Sierra's medium due to the formation of crystals of the calcium salt of lauric acid released by the enzyme. Lipase activity as assayed by measuring the hydrolysis of an emulsified triglyceride (tributyrin) started after 20 d and reached a peak at 44 d. The same pattern of activity was found for DNAase and RNAase (when cultures were grown in phosphate-free medium). No tyrosinase or lysozyme activity was detected.

DISCUSSION

The quantity and turnover rate of microbial biomass accumulated during the preparation and cropping phases of a mushroom compost are unknown. Estimates extrapolated from plate counts and bacterial cell weights indicate that 3–5% of compost dry weight could be bacterial in origin (Fermor & Wood, 1979). Some of the techniques currently employed to

Table 2. *Growth of fungi on autoclaved Bacillus subtilis suspended in solid medium, incubated at 25 °C for 28 d*

Fungal species	Colony radial extension rate* (mm d ⁻¹)	Cleared zone
BASIDIOMYCETES		
<i>Agaricus bisporus</i> (Lange) Imbach D621	0.91	+
<i>Agaricus bisporus</i> (Lange) Imbach S22	0.95	+
<i>Agaricus bitorquis</i> (Quél.) Sacc.	0.87	+
<i>Agaricus macrosporus</i> (Møll. and Schaff.) Pilát	0.72	+
<i>Agaricus silvicola</i> (Vitt.) Peck	0.50	—
<i>Favolus arcularius</i> (Fr.) Ames	1.42	+
<i>Flammulina velutipes</i> (Curt. ex Fr.) Karst	>1.50	+
<i>Pleurotus ostreatus</i> (Jacq. ex Fr.) Kummer	>1.50	+
<i>Stropharia merdaria</i> Fr.	1.28	+
<i>Schizophyllum commune</i> Fr.	>1.50	+
<i>Volvariella volvacea</i> (Bull. ex Fr.) Sing.	>1.50	+
ASCOMYCETES		
<i>Chaetomium globosum</i> Kunze	1.50	+
<i>Diehlomyces microsporus</i> (Diehl and Lambert) Gilkey	NG	—
<i>Neurospora tetrasperma</i> Shear and Dodge	1.90	+
<i>Sordaria fimicola</i> (Roberge) Cesati and de Notaris	2.22	—
FUNGI IMPERFECTI		
<i>Arthrotrichum oligospora</i> Fres.	1.27	+
<i>Aspergillus fumigatus</i> Fres.	1.17	†
<i>Hormiactis alba</i> (Preuss)	<0.50	—
<i>Humicola grisea</i> var. <i>thermoidea</i> Cooney and Emerson	>1.50	+
<i>Penicillium</i> sp.	<0.50	+
<i>Scytalidium thermophilum</i> (Cooney and Emerson) Austwick	<0.50	—
<i>Trichoderma viride</i> Pers.	1.15	+
PHYCOMYCETES		
<i>Mucor hiemalis</i> Wehmeyer	1.55	+
<i>Rhizomucor pusillus</i> (Lindt) Schipper	ND	+
<i>Phycomyces blakesleeana</i> (+) Burgeff, IMI 118496i	NG	—

* ND, Not determined; NG, no growth.

† Very limited.

determine microbial biomass in soils or sediments (Domsch *et al.*, 1979; Paul & Voroney, 1980) are inapplicable to mushroom compost. The measurement of specific cell constituents is invalid because the concentration of these constituents varies according to the proportions of particular organisms in the very heterogeneous population. Techniques which rely on activating a mainly dormant microbial population, such as usually found in soil, and monitoring subsequent metabolic changes have yet to be applied to composts.

There is very little information available on the longevity and fate of bacteria in natural environments. Siala & Gray (1974) observed growth of *B. subtilis* on microscope slides in soil. Growth occurred for 1 week, with a generation time of 9.5 d. In the subsequent 8 weeks the population declined slowly and approximately half the vegetative propagules became spores while the remainder disappeared. In comparison, bacterial numbers and activity in the first two stages of composting are very high (Fermor *et al.*, 1979; Fermor & Wood, 1979). After the 'peak-heating' stage of compost preparation when mushroom mycelium is inoculated into the compost, numbers of bacteria fall initially and then remain at a constant level [10^6 – 10^8 (g dry wt compost)⁻¹; T. R. Fermor, unpublished results] indicating a steady turnover rate.

The population consists of both Gram-positive and Gram-negative bacteria and in the present study *A. bisporus* lysed both. This contrasts with the lytic enzyme *Chalaropsis* B,

Fig. 2. Growth of *A. bisporus* on *B. subtilis* (asporogenous). (a) *B. subtilis* cells cultured in Spizizen salts medium to the late-exponential phase; arrows indicate lysed cells. (b) Cells cultured as in (a), killed in trichloroacetic acid then thoroughly washed in distilled water and extracted with ether before being freeze-dried. (c, d) Cells treated as in (b) suspended in liquid medium and degraded by *A. bisporus* (M) during 35 d growth; arrows indicate cell wall fragments. The bar markers represent 1 μm .

purified by Hash (1963), which lysed Gram-positive bacteria only. Furthermore, Hash did not indicate that the lytic ability of *Chalaropsis* sp. played any part in the nutrition of the fungus. Many of the Basidiomycetes screened here probably produced proteases and possibly also extracellular enzymes capable of hydrolysing the peptidoglycans of bacterial walls (Table 2). Some Ascomycetes, Fungi Imperfecti and Phycomycetes were also able to degrade dead *B. subtilis*, but it would seem that the slower growing Basidiomycetes are more suited to this mode of nutrition. The morphology of Basidiomycetes, compared with that on standard fungal

Fig. 3. Changes in laccase activity (○), dry weight (●) and pH (□) during growth of *A. bisporus* on killed *B. subtilis* in liquid culture.

Fig. 4. (a) Protease and β -N-acetylglucosaminidase activities of *A. bisporus* grown in Treschow basal salts medium containing killed *B. subtilis* as sole carbon and nitrogen source: ●, acid protease (pH 3.6); ■, neutral protease (pH 7.0); ▲, alkaline protease (pH 9.1); ○, β -N-acetylglucosaminidase (pH 5.0).

(b) Protease activities of *A. bisporus* grown in Treschow basal salts medium containing insoluble casein (BDH; 100 g l⁻¹) as sole carbon and nitrogen source; ●, acid protease (pH 3.6); ■, neutral protease (pH 7.0); ▲, alkaline protease (pH 9.1). β -N-Acetylglucosaminidase was not assayed in this medium.

media, was relatively unaltered, although mycelial extension rates were almost halved, whereas Fungi Imperfecti such as *Aspergillus*, *Penicillium* and *Trichoderma*, all renowned for the production of a wide range of extracellular hydrolytic enzymes, showed severely restricted, atypical growth.

Table 3. *Extracellular enzymes assayed in the culture supernatants of A. bisporus growing on killed B. subtilis in Treschow basal salts medium*

Enzyme	EC number	Possible substrate	Presence/ absence	Activity after 40 d
Lysozyme	3. 2. 1. 17	Cell wall	—	—
Laccase	1. 10. 3. 2	(Growth marker)	+	1.75 laccase units ml ⁻¹
Lipase	3. 1. 1. 3	Lipids	+	Not determined
β -N-Acetyl-D-glucosaminidase	3. 2. 1. 30	Cell wall	+	15.0 μ mol nitrophenol min ⁻¹ ml ⁻¹
DNAase	3. 1. 21. 1	DNA	—*	—
RNAase	3. 1. 27. 5	RNA	—*	—
Acid protease (pH 3.6)		Protein	+	10.0 μ g RBB h ⁻¹ ml ⁻¹
Neutral protease (pH 7.0)		Protein	+	37.0 μ g RBB h ⁻¹ ml ⁻¹
Alkaline protease (pH 9.1)		Protein	+	13.0 μ g RBB h ⁻¹ ml ⁻¹
Tyrosinase	1. 14. 18. 1		—	—

* DNAase and RNAase were detected in the supernatants of cultures grown in phosphate-free medium.

In the experiments using *B. subtilis* in liquid cultures it is important to emphasize that the cells were killed, washed and extracted with ether to inactivate endogenous autolysins and to remove the soluble cell contents which would provide an easily assimilated source of nutrition for the fungal mycelium. Thus, the growth observed for *A. bisporus* and other fungi must have been due to the degradation of insoluble polymers.

There did appear to be some material attached to the surface of the bacterial walls; conceivably this was cytoplasmic material released from the cells as a result of their distortion during freeze-drying, and subsequently precipitated and made visible by fixation (Fig. 2*b*). The mode of attack of the lytic enzymes of *A. bisporus* on *B. subtilis* walls parallels the findings of Burdett (1980) who investigated sites of autolysis in *B. subtilis*. Burdett found that most of the autolysin was located at the cross-wall, and following dissolution of the septum, the cylindrical wall was subject to autolysis. Degradation of the bacterial walls in this present study and that of Burdett appeared to occur not by a process of continuous thinning, but by an increase in the size of gaps in the wall and by an increase in the size of holes that were made (Fig. 2*c*). The remaining portions of the wall remained at a relatively constant thickness (Fig. 2*d*).

The extracellular enzymes of *A. bisporus* assayed so far could account for degradation of the bacterial wall polymers and intracellular proteins, lipids and nucleic acids. This fungus has been found to produce an inducible β -N-acetylglucosaminidase but no muramidase; the former enzyme might be significant in peptidoglycan degradation. No tyrosinase activity was found in the culture supernatants showing that all the enzymes assayed were extracellular and not released by autolysis of *A. bisporus* mycelium. Burdett (1980) noted that whatever the specificity of a lysin the morphological sequence of lysis was much the same. For example, *B. subtilis* (with an amidase and glucosaminidase) seemed to be lysed in the same manner as *Lactobacillus* (muramidase). It is likely that *A. bisporus* produces other enzymes capable of degrading bacteria but these have not yet been sought.

The efficiency of conversion of dead bacteria to fungal mycelium and CO₂ was difficult to assess accurately because the mycelium was intimately bound to the bacterial cells on which it was growing and could not be physically separated. However, total dry weight loss in the liquid cultures was 50% in 50 d (Fig. 3), and since a large part of the remaining dry weight consisted of fungal mycelium, the bulk of bacterial biomass had evidently been degraded. Bacterial degradation may, indeed, have been retarded by the clumping of the freeze-dried bacteria used in these experiments.

Wood (1979) found that growth of *A. bisporus* mycelium in liquid cultures was directly proportional to the quantity of extracellular laccase. Thus, the amount of laccase produced in the liquid medium containing bacteria was adopted here as an indication of mycelial growth,

and was found to parallel dry weight loss in the cultures (Fig. 3). The culture pH showed a large shift from acid to alkaline, the cause of which has yet to be determined, but it may have been due to the release of amino groups. The progressive browning of the liquid cultures was probably due to the production of polyphenols by the mushroom mycelium.

These experiments have shown that *A. bisporus* is able to degrade dead bacteria and utilize them as a sole source of both carbon and nitrogen. Bacterial cells may also be capable of acting as sole source of phosphorus, but no nucleases were detected in the standard cultures (Table 3) presumably because the medium had a high phosphate level due to the buffer used. However, DNAase and RNAase activities were detected in cultures containing bacteria as the sole phosphate source. The ability of *A. bisporus* to lyse and degrade live bacteria is at present being determined. Degradation of living or dead bacteria may be a significant mode of nutrition for fungi growing in certain ecological niches, such as leaf litter or other habitats where both bacteria and filamentous fungi are found in quantity. It is noteworthy that slime moulds actively feed on bacteria and they occupy a similar habitat to litter-degrading Basidiomycetes.

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