Introduction

The single largest bioconversion process utilising wood is currently the cultivation of *Lentinus (=Lentinula) edodes*. The Orient cultivates more than US $1 billion of this edible mushroom each year (Royse & Schisler, 1980; San Antonio, 1981; Leatham, 1982). This lignin-degrading basidiomycete (Leatham & Kirk, 1983) is cultivated primarily out of doors on hardwood logs. Growers are now beginning to cultivate this valuable food in other parts of the world (Farr, 1983) and are trying to grow it in environmentally controlled chambers or on new media such as lignocellulosic particles (Hans *et al.*, 1981).

For many growers, however, fruiting is not reliable. The same problem occurs when attempting to cultivate other edible mushrooms under new environmental conditions or on new media. This points to the need for research on the requirements for fungal development and the mechanism by which development is regulated. Unfortunately, the complexity of fungal development, the difficulty in fruiting many of the edible mushrooms in the laboratory, and the frequent use of non-defined media have slowed research progress and limited our understanding of their development.

Recently, a chemically defined medium was developed that rapidly fruits *L. edodes* (Leatham, 1983). Using this medium and a fractionation technique, the author studied the growth and development of *L. edodes*. The areas studied were carbon and nitrogen source uptake and utilisation, soluble protein content, and several extractable enzymes that may be important in development. These results are described here.

Presented first is a brief review of what is currently known about the roles of these enzymes and of carbon and nitrogen source uptake in the growth and development of basidiomycetes including *L. edodes*. This is
followed by a discussion of the new studies with the chemically defined medium.

**Carbon and nitrogen**

Uptake, mobilisation and utilisation of carbon and nitrogen sources are of special importance in the development of fungi. Carbon/nitrogen source ratios can influence whether or not basidiomycetes (Plunkett, 1953; Wessels, 1965) including *L. edodes* (Ando, 1974; Leatham, 1983) produce fruit bodies. It is likely that the carbon and nitrogen sources have separate effects. In a wide range of fungi, nitrogen source depletion precedes and is perhaps essential for the development of fruit bodies. When *L. edodes* was grown on a semi-defined medium, exogenous carbon source was necessary to support the growth of fruit bodies (Tokimoto & Kawai, 1975). Once fruit body growth is underway, rapidly expanding fruit bodies can act as a major sink for carbon and nitrogen compounds and probably other nutrients (Plunkett, 1953; Madelin, 1956; Wessels, 1965; Gruen & Wong, 1982).

**Enzymes**

Organisms require enzymes to utilise nutrients and to regulate growth and development. To carry out its role, each enzyme must be in the appropriate location, i.e. extracellular, cell wall-bound (De Vries & Wessels, 1984), periplasmic, and/or intracellular. To function, each enzyme requires the correct substrate(s) and an acceptable pH. Many classes of enzymes are important to fungal growth and development: those reviewed here include proteases, phosphatases, phenoloxidases, and cell wall-associated polysaccharidases and saccharidases.

Proteases are commonly produced by nitrogen-limited cultures in an attempt to scavenge protein nitrogen (North, 1982). Proteases are developmentally regulated in *Schizophyllum commune* (Schwalb, 1977). During the onset of development in *S. commune* the internal accumulation of proteolytically stable enzymes apparently shifts metabolism from vegetative growth to development (Schwalb, 1978). Proteases are also likely to be involved in the development of other fungi. Protease inhibitors profoundly influence the fruiting of *E. edodes* (Terashita, Kono & Murao, 1978; Terashita et al., 1981).

Phosphatases are developmentally regulated in fungi and myxomycetes (Loomis, 1969; O’Day & Horgen, 1974). Intracellular alkaline phosphatases are present during hyphal tip growth in fungi (Meyer, Parish & Hohl, 1976). Extracellular acid phosphatases are often present in the periplasmic
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space or the cell wall of fungi, yeast and filamentous bacteria (Lampen, 1968; Field & Schekman, 1980; Poirier & Holt, 1983a,b). They are thought to function in cell wall formation (Bojović-Čvetić & Vujčić, 1982; Poirier & Holt, 1983c).

Phenoloxidases, such as laccase, are developmentally regulated in basidiomycetes (Leonard & Phillips, 1973; Wood & Goodenough, 1977; Ross, 1982) including *L. edodes* (Leatham & Stahmann, 1981). Besides polymerising phenols which then form pigments, strengthen cell walls, and protect against pathogens, extracellular phenoloxidases probably have other roles in vegetative growth and development (Leatham & Stahmann, 1981; Leatham, unpublished).

Polysaccharidases and saccharidases have many functions during the growth and development of basidiomycetes. Those relevant to this study include extracellular functions related to basidiomycete cell wall polysaccharides. Water-insoluble (skeletal) cell wall polysaccharides in *basidiomycetes* include chitin microfibrils, (1→3)- and/or (1→6)-β-D-glucans, and (1→2)-α-D-glucans (Rosenberger, 1976; Sietsma & Wessels, 1977; Novaes-Ledieu & Garcia Mendosa, 1981; Wessels & Sietsma, 1981). Water-soluble (non-skeletal) cell wall polysaccharides include homo- or hetero-D-galactans and -D-mannans (Barreto-Bergter & Gorin, 1983). The fruit body cell wall of *L. edodes* contains water-insoluble polysaccharides similar to those described above (Shida, Uchida & Matsuda, 1978; Shida et al., 1981) and a water-soluble L-fuco-D-manno-(1→6)-α-D-galactan (the L-fucose and D-mannose linkages are not yet known; Shida, Haryu & Matsuda, 1975). During hyphal growth or development, synthesis of new cell wall polysaccharides, cross-linking (Sietsma & Wessels, 1979, 1981; Sonnenberg, Sietsma & Wessels, 1982), and lytic modifications in existing cell wall polysaccharides occur (Bartnicki-Garcia, 1973; Gooday & Trinci, 1980; Wessels, Sietsma & Sonnenberg, 1983; Kamada & Takemaru, 1983). Lysis can be extensive. Older cell wall polysaccharides can serve as a source of new cell wall material (Bartnicki-Garcia, 1973; Fèvre, 1977).

Those polysaccharidases and saccharidases produced by basidiomycetes that are capable of degrading insoluble cell wall polysaccharides and/or their intermediate degradation products include laminarinas, β-D-glucosidases, chitinases and *N*-acetyl-β-D-glucosaminidases (Wilson & Niederpreum, 1967; Kamada, Fuji & Takemaru, 1980; Miyake, Takemaru & Ishikawa, 1980; Ishikawa, Oki & Senba, 1983). By analogy, the other appropriate enzymes capable of degrading water-soluble cell wall polysaccharides are probably also produced (e.g. in *L. edodes*: α-D-glucanase, L-fucosidase, D-mannosidase and α-D-galactosidase). An earlier study with
L. edodes grown on a lignocellulosic medium suggested that laminarinase, \( \beta \)-D-glucosidase, \( N \)-acetyl-\( \beta \)-D-glucosaminidase, \( \beta \)-D-mannosidase (but not \( \alpha \)-D-mannosidase) and \( \alpha \)-D-galactosidase were all important during development (Leatham, unpublished).

**Results and discussion**

*Lentinus edodes* ATCC strain 48085 (hetero-dikaryon) was grown on the chemically defined medium developed by Leatham (1983); the composition is given in Table 17.1.

A new fractionation technique was developed to help study the uptake of nutrients and to determine the general location of soluble proteins (Fig. 17.1). The technique was sequential and involved collection of the growth medium, limited homogenisation of the mycelia, and finally extensive homogenisation and breakage of the cells. From these steps were collected the extracellular, shockable, intracellular, and particulate fractions. Water used during the homogenisations extracted soluble components and, by dilution of residual components, resulted in minimal contamination of subsequent fractions. What follows is an explanation of how the technique fractionates and the likely sources of the components in each fraction.

Vacuum filtration of whole cultures on glass fibre filters did not extract the aerial mycelia nor damage the cultures. Thus, the extracellular fraction contained components in the growth medium and also extracellular metabolites and enzymes secreted into it. Limited homogenisation of the mycelium in a Waring blender effectively wetted the extracellular surfaces of the aerial mycelium and only partially fragmented the mycelium. Thus, the shockable fraction contained components extracted from the aerial mycelium and components released during partial fragmentation, i.e. from the following locations: cell wall, periplasmic space, cell membrane (extracellular side), fragile cells (e.g. hyphal tips), and vesicles accumulated near the cell membrane (e.g. undergoing extracellular transport). Cell breakage of the mycelial fragments in the Braun homogeniser was essentially complete. Thus, the intracellular fraction contained components extracted only after extensive cell breakage. The final vacuum filtration of the final homogenate collected insoluble cell debris. Thus, the particulate fraction contained components not previously extracted (solubilised). No attempt was made to fractionate further (or characterise) the particulate fraction (Poirier & Holt, 1983c).

In this technique there was obvious concern as to the extent of cell breakage during preparation of the shockable fraction – this would result
Results and discussion

Table 17.1. Composition of the chemically defined medium used to grow and fruit Lentinus edodes (Leatham, 1983)

<table>
<thead>
<tr>
<th>Component</th>
<th>Final concentration (per litre)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Glucose</td>
<td>25.0 g</td>
</tr>
<tr>
<td>D-Glucuronic acid</td>
<td>4.0 g</td>
</tr>
<tr>
<td>L-Glutamic acid</td>
<td>2.5 g</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>2.0 g</td>
</tr>
<tr>
<td>MgSO$_4$·7H$_2$O</td>
<td>2.0 g</td>
</tr>
<tr>
<td>MnSO$_4$·5H$_2$O</td>
<td>43.9 mg</td>
</tr>
<tr>
<td>CaCl$_2$·2H$_2$O</td>
<td>36.7 mg</td>
</tr>
<tr>
<td>ZnSO$_4$·7H$_2$O</td>
<td>22.0 mg</td>
</tr>
<tr>
<td>Fe(NH$_4$)$_2$(SO$_4$)$_2$·6H$_2$O</td>
<td>14.1 mg</td>
</tr>
<tr>
<td>CuSO$_4$·6H$_2$O</td>
<td>784.0 µg</td>
</tr>
<tr>
<td>CoCl$_2$·2H$_2$O</td>
<td>81.0 µg</td>
</tr>
<tr>
<td>NiCl$_2$·6H$_2$O</td>
<td>81.0 µg</td>
</tr>
<tr>
<td>Na$_2$MoO$_4$·2H$_2$O</td>
<td>51.0 µg</td>
</tr>
<tr>
<td>SnCl$_2$·2H$_2$O</td>
<td>38.0 µg</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>1.0 mg</td>
</tr>
<tr>
<td>i-Inositol</td>
<td>1.0 mg</td>
</tr>
<tr>
<td>Thiamine HCl</td>
<td>1.0 mg</td>
</tr>
<tr>
<td>p-Aminobenzoic acid</td>
<td>100.0 µg</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>100.0 µg</td>
</tr>
<tr>
<td>Pyridoxine HCl</td>
<td>100.0 µg</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>100.0 µg</td>
</tr>
<tr>
<td>Sodium pantothenate</td>
<td>100.0 µg</td>
</tr>
<tr>
<td>Biotin</td>
<td>30.0 µg</td>
</tr>
<tr>
<td>Cyanocobalamin</td>
<td>10.0 µg</td>
</tr>
<tr>
<td>Folic acid</td>
<td>10.0 µg</td>
</tr>
</tbody>
</table>

a Distilled/deionised water was used as solvent. The medium was adjusted to pH 4.0 with KOH, filter sterilised through 0.22 µm-size filters, and stored at 4 °C until use.

b These unusual trace elements were added because they stimulate fruiting (Leatham, 1985).

in intracellular components contaminating the shockable fraction. By using an intracellular component as a marker, it was possible to approximate an upper limit for the extent of cell breakage. In doing so it was necessary to assume that (1) all of the protein in the shockable fraction was derived from cell breakage, and (2) young and middle-aged mycelia were equally fragile. Based on protein content in the intracellular and shockable fractions from 6- and 34-day-old cultures, apparently no more than 23%
cell breakage occurred during preparation of the shockable fractions. Thus, unless a residual component was incompletely extracted, the shockable and other fractions were at least 77% free of components from other culture fractions.

Fig. 17.1. Technique for fractionating whole cultures grown in the chemically defined medium. Homogenisations and cell breakage were in distilled/deionised water at 1-5°C. Homogenisations were at low speed in an Eberbach model 8580 semi-micro stainless steel blender assembly and Waring blender using 25 ml total sample volume per 25-ml culture. Cell breakage was at 4000 rpm in a Braun 70-ml glass homogenising cell and Braun model MSK homogeniser using 30 ml total sample volume and 20 ml of 0.1-0.2-mm-diameter glass beads per culture. Glass fibre filters were used during all vacuum filtrations to collect mycelial fragments or to remove cell fragments and glass beads from filtrates.
Results and discussion

Growth, growth stages and development

Analysis of growth and development of *L. edodes* on the chemically defined medium has revealed the growth stages and extent of development.

Fig. 17.2. Growth, growth stages and the development of cultures of *L. edodes* in a chemically defined medium (Leatham, 1983). Cultures were incubated at 22 ± 1 °C and 80% relative humidity with illumination at 200–400 lux from 40-watt Sylvania Gro-Lux fluorescent bulbs on a 9 h light/15 h dark cycle (Leatham & Stahmann, 1981). **Upper graph:** values for growth are expressed as the log of the average dry weight (mg) for mycelia from triplicate cultures dried at 60 °C. **Middle graph:** changes in growth were used to assign the growth stages. **Lower graph:** growth stages are delineated (vertical dotted lines). Development was determined by visual inspection of the cultures.
Growth & development of *Lentinus edodes* of the fungus at different ages (Fig. 17.2). Early in the vegetative growth stage, only submerged mycelia grew, aerial mycelia forming later. During the transition stage, the rate of growth slowed to one-half and then to one-fourth of the initial rate. In spite of this, before growth finally stopped the mycelial weight had doubled during the transition stage.

Droplet exudation from tiny primordia buried within the mycelium was the first outward sign of development. All cultures exuded droplets. Exudation began on day 18 (during the transition stage) and was most active through day 30. Following the active exudation period, approximately 40% of the cultures fruited. Fruiting began on day 25 (near the end of the transition stage) and continued through day 50. The bulk of fruiting usually happened at two times - near days 27 and 36 - but was most frequent near day 36. Each culture usually fruited only once and produced one or two fruit bodies. Although other possibilities exist, this pattern suggests that *L. edodes* may have fruited as a result of the depletion of an essential nutrient.

Once fruiting was initiated, fruit body expansion was rapid and a considerable portion of the total fungal mass was committed to fruit body formation. Fruit bodies produced by individual cultures commonly grew to full weight within 2–5 days and contained up to 48% of the total colony dry weight.

**Uptake and utilisation of carbon source**

D-Glucose is the major carbon source in the chemically defined medium. Throughout the transition stage, at least 20% of the initial D-glucose remained in the extracellular fraction (Fig. 17.3). Only near day 45 was the D-glucose depleted. These observations have important implications. Carbon limitation does not trigger either the transition stage or primordia formation. However, as Tokimoto & Kawai suggested (1975), depletion of carbon source perhaps best explains the failure of cultures to fruit. Since primordia produce fruit bodies by expansion, it is probable that carbon limitation stops development by blocking primordium expansion.

*L. edodes* is efficient in utilising D-glucose as a carbon source. The maximal theoretical yield of cells for an aerobic organism grown on glucose is near 0.5 g cells g⁻¹ glucose consumed (Righelato, 1975). Here, the cell yield of *L. edodes* on D-glucose was near the theoretical limit. At the end of the vegetative growth stage (day 16), 0.6 g of cells had been produced per gram of D-glucose consumed (Fig. 17.3). At the time of
Fig. 17.3. Uptake and utilisation of carbon and nitrogen sources by cultures grown in the chemically defined medium. *Upper graph:* carbon source uptake was determined by measuring the amount (%) of D-glucose remaining in the extracellular fraction (solid line) of duplicate cultures with a Beckman Model 2 Glucose Analyzer equipped with a D-glucose oxidase/O2 probe using D-glucose as standard. Cell yields (g cells formed g−1 glucose utilised) in cultures (dashed line) were calculated using the growth data in Fig. 17.2. *Lower graph:* nitrogen source uptake was determined by measuring the amount (%) of total nitrogen (initially L-glutamate) remaining in the extracellular fraction (solid line) of duplicate cultures by the micro-Kjeldahl procedure using acetanilide as standard. The nitrogen contents of cells (g nitrogen utilised g−1 cells formed) in the cultures (dashed line) were calculated using the growth data in Fig. 17.2. Growth stages (see Fig. 17.2) are delineated (vertical dotted lines).
D-glucose depletion (day 45), 0.39 g of cells had been produced per gram of D-glucose consumed.

**Uptake and utilisation of nitrogen source**

L-Glutamic acid is the only significant nitrogen source in the chemically defined medium. In contrast to D-glucose, L-glutamic acid was rapidly depleted from the extracellular fraction (Fig. 17.3). By the beginning of the transition stage (day 20), more than 90% of the total extracellular nitrogen had been taken up by the mycelium. Because the nitrogen level did not change after day 20, the L-glutamic acid was probably depleted. The nitrogen remaining in the medium after day 20 was probably fungal protein. Based on these observations, it is likely that nitrogen source depletion triggered the transition stage. Because of the depletion of utilisable extracellular nitrogen, growth during the transition stage might occur at the expense of stored nitrogen reserves, which perhaps includes nitrogen liberated from protein and/or nucleic acids (Legerton & Weiss, 1979).

The medium composition is known to influence the efficiency by which certain strains of *L. edodes* utilise L-glutamic acid as a nitrogen source (Kawamura & Goto, 1980). With the medium used here, this strain of *L. edodes* utilised L-glutamic acid efficiently. Fungal cells are characteristically 4-7% nitrogen by dry weight. In the current study, the cell yield of *L. edodes* was remarkably high. At the end of the vegetative growth stage through the onset of the transition stage (days 16–20) the cells contained 4% nitrogen (Fig. 17.3). Due to the approximate doubling in weight (and the absence of available extracellular nitrogen source) during the transition stage (day 16–30), this value decreased until it reached 2.2% nitrogen. Failure to grow further suggests depletion of stored nitrogen source reserves.

These results indicate that development is likely to be regulated by nitrogen limitation. Droplet exudation occurred as the utilisable extracellular nitrogen was depleted. Fruiting occurred with the probable depletion of reserve nitrogen sources. These results also explain how carbon: nitrogen source ratios may regulate development in basidiomycetes that produce abundant or large fruit bodies. Adequate nitrogen source is undoubtedly required to produce a mycelium of sufficient mass to support development. However, nitrogen source depletion is not possible without sufficient carbon source to utilise the nitrogen source. Carbon: nitrogen source ratios may also be important to fungi such as *L. edodes* growing on wood. The
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low nitrogen content of wood undoubtedly ensures rapid nitrogen limitation.

Protein production and localisation and resistance to proteases

A mechanism is needed to explain how nitrogen limitation may regulate the development of *L. edodes*. Because the patterns for the production, stability and turnover of protein may be involved, they were investigated. With the chemically defined medium, maximum soluble protein was produced during the vegetative growth stage (Fig. 17.4). Consistent with the low nitrogen source content of the medium, little protein accumulated in the extracellular fraction. Instead, the majority accumulated in the intracellular and shockable fractions. Nearly equivalent amounts of protein were in these two fractions. The exact cellular location and function(s) of the protein are not known and deserve study. Recently,

Fig. 17.4. Production and localisation of protein in cultures grown in the chemically defined medium. The protein contents of cultures were determined on fractions from triplicate cultures by the Coomassie blue dye method (Spector, 1978). Values are given for the total protein contents per culture (mg) in the extracellular (dotted line), shockable (dashed line) and intracellular (solid line) fractions. Growth stages (see Fig. 17.2) are delineated (vertical dotted lines).
De Vries and Wessels (1984) suggested that cell wall protein in *S. commune* is involved in fruiting.

With the onset of the transition stage in *L. edodes* (day 16), a marked loss in total soluble protein occurred (Fig. 17.4). As the utilisable extracellular nitrogen was depleted (Fig. 17.3), protein levels in the intracellular and shockable fractions decreased to 20% and 11% of their

![Graph](image)

Fig. 17.5. Accumulation of glucosamine by nitrogen-limited cultures during degradation of a shredded oak wood/oatmeal medium. **Upper graph**: total dry weights (%) remaining after different lengths of incubation were determined on triplicate whole cultures (medium and fungus) dried at 60 °C (solid line). Extractable protein (%) remaining was determined in distilled-water extracts from triplicate cultures by the Coomassie blue dye method (Spector, 1978) (dotted line). **Lower graph**: d-glucosamine contents were determined on acid-hydrolysed samples from pooled triplicate cultures by the method of Gurusiddaiah, Blanchette & Shaw (1978).
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original levels, respectively. In contrast, the low protein level in the extracellular fraction remained nearly constant. These data suggest that a massive reorganisation of soluble protein occurs during the transition stage. Late in the life cycle, enzymes essential for vegetative growth are certainly not as important as are enzymes essential for fruiting. Proteases and other developmentally regulated enzymes are likely to be induced in these nitrogen-depleted cultures. Thus, only those proteins that are either stable to proteolysis or rapidly resynthesised will accumulate.

Because these data suggest that significant net protein turnover occurs during the transition stage, it is important to determine the fate of the nitrogen from scavenged protein. Major non-protein sinks for nitrogen in developing fungi may include DNA for spore (nucleus) formation. D-glucosamine for chitin (cell wall) synthesis, or urea for osmotic pressure (needed for fruit body pileus expansion in *Coprinus cinereus* (Moore, Elhiti & Butler, 1979)). When cultures of *L. edodes* were grown on a lignocellulosic medium (Leatham, unpublished), analysis of acid-hydrolysed samples showed that after depletion of utilisable nitrogen source (protein), the cultures rapidly accumulated D-glucosamine, resulting in a net five-fold increase (Fig. 17.5). The form of the D-glucosamine is not yet known but it may be in reserve for the later production of fruit bodies (Tokimoto & Fukuda, 1981). If the same process occurs in cultures grown on the chemically defined medium, this may account for a significant amount of the soluble protein lost during the transition stage. However, for protein turnover and development to occur, the proteases and the other necessary enzymes must be present.

Production, localisation and possible roles of proteases.

Proteases. Proteases are produced by *L. edodes* and are in distinct locations. Cultures began to exhibit significant protease activities late in the vegetative growth stage (Fig. 17.6). These peaked during the transition stage near day 20 (after nitrogen source depletion had occurred) and were in high titres throughout the rest of the incubation. Proteases with pH optima acceptable for *in vivo* function were located in each of the culture fractions. The extracellular fraction contained acid protease but little detectable neutral or alkaline protease. The intracellular fraction contained neutral and alkaline proteases but little detectable acid protease. The shockable fraction contained all three classes of protease. Essentially non-synchronous fluctuations in protease titres began during the transition stage and the extracellular acid protease peaked near the end of the
incubation. These data suggest that the three classes of proteases are distinct and that their titres are developmentally regulated. The appearance, relative abundance and widespread localisation of proteases suggest that significant rates of protein turnover are indeed possible in nitrogen-depleted cultures.

As reported for *S. commune* (Schwalb, 1977, 1978), protein turnover and
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Table 17.2. Stability of total protein and enzymes in fractions from 24-day-old cultures of Lentinus edodes during storage for 4 days at 4 °C

<table>
<thead>
<tr>
<th>Component</th>
<th>Percent of original remaining in the culture fraction after storage</th>
<th>pH of assay</th>
<th>Extracellular fraction</th>
<th>Shockable fraction</th>
<th>Intracellular fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total protein</td>
<td>95</td>
<td></td>
<td></td>
<td>31&lt;sup&gt;si&lt;/sup&gt;</td>
<td>45&lt;sup&gt;sii&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Enzymes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acid protease</td>
<td>4.0</td>
<td>109&lt;sup&gt;ii&lt;/sup&gt;</td>
<td>96&lt;sup&gt;i&lt;/sup&gt;</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Neutral protease</td>
<td>7.0</td>
<td>NA</td>
<td>108&lt;sup&gt;i&lt;/sup&gt;</td>
<td>89&lt;sup&gt;i&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Alkaline protease</td>
<td>8.5</td>
<td>NA</td>
<td>108&lt;sup&gt;i&lt;/sup&gt;</td>
<td>136&lt;sup&gt;i&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>4.0</td>
<td>102</td>
<td>93&lt;sup&gt;i&lt;/sup&gt;</td>
<td>13&lt;sup&gt;i&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Neutral phosphatase</td>
<td>7.0</td>
<td>NA</td>
<td>72&lt;sup&gt;ii&lt;/sup&gt;</td>
<td>29&lt;sup&gt;i&lt;/sup&gt;</td>
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<tr>
<td>Alkaline phosphatase</td>
<td>8.5</td>
<td>NA</td>
<td>57</td>
<td>63&lt;sup&gt;i&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Laccase</td>
<td>4.0</td>
<td>98&lt;sup&gt;i&lt;/sup&gt;</td>
<td>65&lt;sup&gt;i&lt;/sup&gt;</td>
<td>59</td>
<td></td>
</tr>
<tr>
<td>β-D-Glucosidase</td>
<td>4.0</td>
<td>74&lt;sup&gt;i&lt;/sup&gt;</td>
<td>81&lt;sup&gt;i&lt;/sup&gt;</td>
<td>48&lt;sup&gt;i&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>N-Acetyl-β-D-</td>
<td>4.0</td>
<td>81&lt;sup&gt;i&lt;/sup&gt;</td>
<td>101&lt;sup&gt;i&lt;/sup&gt;</td>
<td>78</td>
<td></td>
</tr>
<tr>
<td>Glucosaminidase</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-D-Galactosidase</td>
<td>4.0</td>
<td>92</td>
<td>101&lt;sup&gt;i&lt;/sup&gt;</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>β-D-Mannosidase</td>
<td>4.0</td>
<td>82</td>
<td>97&lt;sup&gt;i&lt;/sup&gt;</td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> The final average pH of the culture fractions were 3.7, 5.4 and 9.3, respectively. Endogenous proteases are present in all fractions. Pooled triplicate culture fractions were assayed.

<sup>b</sup> Determined by the Coomassie blue dye method (Spector. 1978).

<sup>c</sup> Methods for enzyme assays are given in the legends for Figs 17.6 through 17.10. NA: Activity normally not present in this culture fraction.

<sup>d</sup> Dominant site for this particular protein/enzyme.

<sup>e</sup> Marked instability of the protein/enzyme in this culture fraction. Protease inhibitors decrease the extent of net protein disappearance during storage.

The relative stability of enzymes to proteolysis may be important to the development of *L. edodes*. Studies on protein and enzyme stability in fractions from nitrogen-depleted cultures stored for 4 days at 4 °C showed that both the total soluble protein and specific enzymes in the extracellular fraction were resistant to proteolysis by endogenous proteases (Table 17.2). In contrast, the bulk of the total protein in the shockable and intracellular fractions was not stable during storage. 55–69% of it was apparently degraded. Even so, several enzymes in these latter two fractions were markedly stable, especially when in the culture fraction of their dominance. These included proteases, phosphatases, laccase, and certain saccharidases (Table 17.2). This stability may help account for their relatively high titres in these nitrogen-depleted cultures.
Fig. 17.7. Production and localisation of acid (upper graph), neutral (middle graph), and alkaline phosphatases (lower graph) in cultures grown in the chemically defined medium. Titres were determined at 22 °C on fractions from triplicate cultures in 50mM pH 4.0, 7.0, or 8.5 buffer (sodium acetate (HCl), piperazine-\(N,N'\)-bis[2-ethane-sulphonic acid] (NaOH), or tri(hydroxymethyl)amino-methane (HCl), respectively) with 3.3mM \(p\)-nitrophenol-phosphate ester as substrate (increase in \(A_{400}\)). Values are given for the total enzyme units (international units) per culture in the extracellular (dotted lines), shockable (dashed lines), or intracellular (solid lines) fractions. Growth stages (see Fig. 17.2) are delineated (vertical dotted lines).
Proteases are known to have functions in addition to protein turnover. Regulation by proteolytic activation of enzymes is important in many eukaryotes (Marzluf, 1981). Chitin synthetases of yeast (Cabib & Ulane, 1973) and basidiomycetes (e.g. *Agaricus bisporus* (Hänseler, Nyhlén & Rast, 1983)) are known to be proteolytically activated. Many proteins produced by developing cultures of *S. commune* are known to undergo post-translational modification (De Vries *et al.*, 1980). Certainly some may undergo proteolytic modifications including proteolytic activation. However, other modifications are also possible including glycosylation and phosphorylation. Yeast fructose-1,6-bis-phosphatase, a key metabolic enzyme, is regulated by proteolytic cleavage and by phosphorylation/dephosphorylation (Tortora *et al.*, 1981).

**Phosphatases.** Phosphatases are produced by *L. edodes* and are in distinct locations (Fig. 17.7). Like the protease activities, phosphatase activities became significant late in the vegetative growth stage and remained in high titre throughout the rest of the incubation period. The phosphatase in highest titre was acid phosphatase, followed by moderate and low titres of neutral and alkaline phosphatases, respectively. Unlike the proteases, relatively little phosphatase was found in the extracellular fraction. Acid and neutral phosphatases were located predominantly in the shockable fraction. Alkaline phosphatase was located predominantly in the intracellular fraction and was at least 20 times lower in titre than the acid or neutral phosphatases. The titres of acid and alkaline (but not neutral) phosphatases underwent marked fluctuations. Acid phosphatase titres fluctuated during the transition stage and afterwards, whereas alkaline phosphatase titres fluctuated significantly only during the transition stage. These data suggest that, like the proteases, the three classes of phosphatases are both distinct and developmentally regulated.

As discussed earlier, little is known about the roles for phosphatases in fungi. These studies suggest that the phosphatases of *L. edodes* have more than one role. Since the extracellular pH of the primordia and fruit bodies of *L. edodes* is maintained near 4.0 (Leatham & Stahmann, 1981), it is likely that the relatively abundant acid phosphatase has a role in the cell wall region. The natural substrate(s) for this enzyme is not known and thus deserves study. Perhaps phosphoprotein, phospho-β-D-glucan (e.g. mycolaminarin in *Phytophthora* (Wang & Bartnicki-Garcia, 1980)), or another phosphopolysaccharide (e.g. phospho-α-D-mannan in *Hansenula* (San-Blas & Cunningham, 1974)) is the natural substrate.
Laccase. Laccase active at pH 4.0 is produced by cultures of *L. edodes* grown on the chemically defined medium (Fig. 17.8). Confirming earlier work with cultures grown on non-defined media (Leatham & Stahmann, 1981), the laccase is in distinct locations, and the titre in each location changes with the growth stage. Laccase was initially excreted into the medium. Later, as the (non-pigmented) aerial mycelium formed, the laccase titre increased in the shockable fraction. The greatest increase occurred during formation of the pigmented primordia and fruit bodies. Because the laccase was extracellular in these aerial structures, it was found predominantly in the shockable fraction. These data suggest that the laccase, too, is developmentally regulated and, as discussed earlier (Leatham & Stahmann, 1981), plays an extracellular role in the cell wall region. The

![Fig. 17.8. Production and localisation of laccase in cultures grown in the chemically defined medium. Titres were determined at 22 °C on fractions from triplicate cultures in 50mM pH 4.0 sodium acetate (HCl) buffer with 1mM o-toluidine as substrate (increase in A, Leatham & Stahmann, 1981). Values are given for the total enzyme units (international units) per culture in the extracellular (dotted line), shockable (dashed line), and intracellular (solid line) fractions. Growth stages (see Fig. 17.2) are delineated (vertical dotted lines).](image-url)
current study shows that intracellular roles are unlikely; little laccase was detected in the intracellular fraction at any growth stage (Fig. 17.8).

**Saccharidases.** Saccharidases active at pH 4.0 are produced by *L. edodes* and are in distinct locations (Figs 17.9 and 17.10). They included β-D-glucosidase, N-acetyl-β-D-glucosaminidase, α-D-galactosidase and β-D-mannosidase. As with the proteases, phosphatases and laccase, saccharidase titres became significant late in the vegetative growth stage and remained high throughout the incubation period. The enzyme in

![Graph](image)

Fig. 17.9. Production and localisation of β-D-glucosidase (upper graph) and N-acetyl-β-D-glucosaminidase (lower graph) in cultures grown in the chemically defined medium. Titres were determined at 22 °C on fractions from triplicate cultures in 50mM pH 4.0 sodium acetate (HCl) buffer with 3.3mM *p*-nitrophenol-β-D-glucopyranoside or *p*-nitrophenol-2-acetamido-2-deoxy-β-D-glucopyranoside as substrate, respectively (increase in $A_{400}$). Values are given for the total enzyme units (international units) per culture in the extracellular (dotted lines), shockable (dashed lines) and intracellular (solid lines) fractions. Growth stages (see Fig. 17.2) are delineated (vertical dotted lines).
highest titre was β-D-glucosidase, followed by N-acetyl-β-D-glucosaminidase. During the transition period, the saccharidases were in highest titre in the shockable fraction. After the transition period, however, their titres also increased in the extracellular fraction. With the exception of β-D-mannosidase, the saccharidase titres all markedly increased as the D-glucose (carbon source) in the growth medium became depleted (compare Fig. 17.3 with Figs 17.9 and 17.10). During all growth stages only low and moderately low activities of β-D-glucosidase and N-acetyl-β-D-glucosaminidase, respectively, were found in the intracellular fraction. In contrast,
Summary and conclusions

essentially no α-D-galactosidase or β-D-mannosidase activity was present in the intracellular fraction (Fig. 17.10). Based on these data it is possible that these saccharidases have roles in the cell wall of _L. edodes_. Their natural substrates may be the insoluble cell wall β-D-glucans, chitin and the water-soluble L-fuco-D-manno-α-D-galactan of _L. edodes_, or their partial degradation products. The low _N_‑acetyl-β-D-glucosaminidase and β-D-glucanase activities in the intracellular fraction may be important. Chitin microfibrils are the innermost structural polymer in the cell wall of _L. edodes_ and they are embedded in extensively branched β-D-glucans (Shida _et al._, 1981). During collection of the shockable fraction these enzymes may be incompletely extracted from these insoluble substrates.

Summary and conclusions

The studies summarised above have shown that during growth on the chemically defined medium, _L. edodes_ (1) efficiently uses D-glucose as carbon source; (2) efficiently uses L-glutamic acid as nitrogen source; (3) depletes the nitrogen source before the carbon source; (4) enters a transition stage probably triggered by nitrogen source depletion; (5) produces proteases; (6) slows in growth rate; (7) loses more than 80%; of its total soluble protein; (8) produces and accumulates protease-resistant enzymes in specific locations, with appropriate pH optima, including proteases, phosphatase, laccase, β-D-glucosidase, _N_‑acetyl-β-D-glucosaminidase, α-D-galactosidase and β-D-mannosidase; and (9) produces fruit bodies as net growth (increased dry mass) finally stops. Based on these observations, it is likely that nitrogen limitation regulates development in _L. edodes_ and that, in part, the regulatory mechanism involves the new synthesis and the proteolytic turnover and/or modification of key enzymes.

The sequential fractionation technique developed for this study was effective in localising soluble components in the extracellular, shockable or intracellular fractions. Those in the shockable fraction clearly merit further study. During the vegetative growth stage, half of the total soluble protein was in the shockable fraction. Afterwards, acid phosphatase, laccase, β-D-glucosidase, _N_‑acetyl-β-D-glucosaminidase, α-D-galactosidase and β-D-mannosidase were in highest titre in the shockable fraction. These enzymes presumably were extracted from the cell wall region and all may have roles related to development.

Further use of fractionation/localisation techniques and chemically defined media will facilitate research into the roles of these and other enzymes in fungal development. The practical application of this knowledge
should result in more reliable industrial methods for producing edible mushrooms.

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**References**


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