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Extracellular Enzymes Produced by the Cultivated Mushroom *Lentinus edodes* during Degradation of a Lignocellulosic Medium

GARY F. LEATHAM

U.S. Department of Agriculture/Forest Service, Forest Products Laboratory, Madison, Wisconsin 53705

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Although the commercially important mushroom *Lentinus* (= *Lentinula*) *edodes* (Berk.) Sing. can be rapidly cultivated on supplemented wood particles, fruiting is not reliable. This study addressed the problem by developing more information about growth and development on a practical oakwood-oatmeal medium. The study determined (i) the components degraded during a 150-day incubation at 22°C, (ii) the apparent vegetative growth pattern, (iii) the likely growth-limiting nutrient, and (iv) assays that can be used to study key extracellular enzymes. All major components of the medium were degraded, lignin selectively so. The vegetative growth rate was most rapid during the initial 90 days, during which weight loss correlated with glucosamine accumulation (assayed after acid hydrolysis). The rate then slowed; in apparent preparation for fruiting, the cultures rapidly accumulated glucosamine (or its oligomer or polymer). Nitrogen was growth limiting. Certain enzyme activities were associated with the pattern of medium degradation, with growth, or with development. They included cellulolytic system enzymes, hemicellulases, the ligninolytic system, (gluco-)amylase, pectinase, acid protease, cell wall lytic enzymes (laminarinase, 1,4-β-D-glucosidase, β-*N*-acetyl-D-glucosaminidase, α-D-galactosidase, β-D-mannosidase), acid phosphatase, and laccase. Enzyme activities over the 150-day incubation period with and without a fruiting stimulus are reported. These results provide a basis for future investigations into the physiology and biochemistry of growth and fruiting.

The single largest bioconversion process utilizing wood is the cultivation of the edible shiitake mushroom (*Lentinus edodes*). In Japan alone, over 2×10^6 cubic meters of wood are converted annually into over $1 billion (U.S.) worth of this food (21). Interest in the commercial cultivation of this mushroom is now rapidly expanding in the United States. This white-rot (22, 26, 38) basidiomycete is commonly cultivated outdoors on oak logs, where for years after inoculation it fruits during cool wet periods (21, 30). The fungus is less commonly cultivated in environmentally controlled chambers on wood or other lignocellulosic particles supplemented with a cereal grain or bran (16). When compared with cultivation on logs, cultivation on particles can give higher yields in months instead of years. Unfortunately, this more intensive cultivation process is currently unreliable. A better knowledge of the physiology and biochemistry of solid-substrate degradation by *L. edodes* is needed to develop better growth media, to optimize cultivation techniques, to predict fruiting cycles, and to detect potential crop failures. This study addressed this need.

A practical oakwood-oatmeal medium was chosen for the study. *L. edodes* was cultivated on the medium for 150 days at 22°C. During that time changes in medium composition were followed, vegetative growth was monitored, and selected enzyme activities which relate to substrate degradation, to growth, and to development were determined. A fruiting stimulus (cold treatment) was given to some 90-day-old cultures to help identify enzymes important to fruiting. The results provide background information needed for further research aimed at understanding the degradative abilities of this white-rot fungus and improving the reliability of its fruiting.

MATERIALS AND METHODS

Chemicals. Chemicals were obtained from the following sources: U-ring-^{14}C-synthetic lignin (18) was provided by T. Kent Kirk of this Laboratory; glucuronoxylan, prepared by KOH extraction of aspen (*Populus tremuloides* Michx.) wood was provided by R. W. Scott of this Laboratory; glucomannan and arabinoglucuronoxylan from larch (*Larix occidentalis* Nutt.) wood xylan (4) (P&L Biochemicals, Inc., Milwaukee, Wis.) was provided by T. L. Highley of this Laboratory; Solka-floc SW-40 (a wood pulp cellulose) was from Brown Co. (Berlin, N.H.); cotton was from Absorbant Cotton Co. (Valley Park, Mo.); Avicel (microcrystalline cellulose) was from FMC Corp. (Marcus Hook, Pa.); gum karaya, gum tragacanth, locust bean gum, salicin, and glucosamine hydrochloride were from ICN Nutritional Biochemicals (Cleveland, Ohio); gum arabic was from Stein Hall & Co., Inc. (New York, N.Y.); *o*-tolidine dihydrochloride was from Eastman Kodak Co. (Rochester, N.Y.); *p*-nitrophenol (PNP)-α-D-maltoside was from Calbiochem-Behring (La Jolla, Calif.); PNP-α-D-xylopyranoside was from Koch-Light Laboratories (Haverhill, United Kingdom); PNP-α-L-arabinofuranoside, other PNP analogs, PNP, amylopectin, amylose, high-viscosity carboxymethylcellulose, cellulose azure, laminarin, chitin (from crab shell), yeast cell wall α-mannan, poly-1,4-α-D-galacturonic acid, sucrose, hide-powder azure, and bovine serum albumin (fraction V) were from Sigma Chemical Co. (St. Louis, Mo.); 3,5-di-*t*-butylbenzoquinone was from Aldrich Chemical Co. (Milwaukee, Wis.).

Fungus, media, growth conditions, and fruiting stimulus. Cultures of *L. edodes* heterodikaryon ATCC 48085 were maintained at room temperature on Sabouraud dextrose agar (Difco Laboratories, Detroit, Mich.) slants. The lignocellulosic medium consisted of 10 g (dry weight) of debarked fresh northern red oak (*Quercus rubra* L.) wood shredded to approximately 20- by 3- by 0.5-mm pieces, 8 g (dry weight) of rolled oats, and distilled-deionized H_2O added to obtain 60% final moisture. The medium, in cotton-stoppered 125-ml Erlenmeyer culture flasks, was sterilized by autoclaving at 121°C for 1 h. Inoculum was produced by transferring mycelium to aluminum foil-capped flasks containing moist,

whole oat kernels (50% [wt/wt] H_2O) which had been sterilized by autoclaving at 121°C for 1.5 h. After 21 days of incubation at room temperature, three kernels were used to inoculate each flask. Cultures were incubated at 22°C and 80% relative humidity with 430 lx of light from Gro-Lux fluorescent bulbs (Sylvania Co., Danvers, Mass.) on a 9-h light–15-h dark cycle. A fruiting stimulus was given to some 90-day-old cultures: a 12-day cold treatment at 5°C followed by 3 days at 22°C.

Culture composition determinations. Dry weights and moisture contents of the samples were determined by drying at 60°C. Culture pH was measured with 5-g (wet weight) samples of freshly harvested cultures after suspending in 20 ml of deionized H_2O for 30 min. Values for dry weight, moisture content, and pH are expressed as mean ± standard deviation for triplicate cultures.

Chemical analyses were on dried samples ground to 40-mesh size. Total sugars (8), individual sugars (41), uronic anhydrides (31), lignin (8), and glucosamine (2-h hydrolysis) (14) were determined after acid hydrolysis. Values for sugar and lignin content are reported as mean ± standard deviation for duplicate cultures. Glucosamine values are reported as single determinations on pooled triplicate cultures (no correction was made for glucosamine loss). Glucosamine recovery from glucosamine hydrochloride or chitin added to the samples was 68.0 and 37.6% respectively.

Ligninolytic activity assay. Ligninolytic activity was assayed at in vivo pH by the method of Wood and Leatham (46) with an aqueous suspension of U-ring-^{14}C-synthetic lignin (50 μg [45,000 dpm]/ml) as the substrate (18, 20). To assay, 5-g (fresh weight) samples of the contents from each freshly harvested culture were placed into duplicate 125-ml Erlenmeyer flasks. A 2-ml sample of the lignin suspension was dispensed uniformly over each sample, and the cultures were then incubated under O_2 for 60 days at 22°C with periodic flushing with O_2 and trapping of $^{14}CO_2$ (18, 20). The percentage of the total ^{14}C evolved as $^{14}CO_2$ for the duplicate assays were averaged, and results are reported as the mean ± standard deviation for triplicate cultures (total of six replicates).

Culture extraction and extractable protein determination. Individual cultures were suspended in 75 ml of deionized H_2O and incubated at room temperature for 2 h with 10-s stirring every 15 min. The culture extracts were filtered through nylon mesh and then glass wool and centrifuged at 10,000 × *g* for 10 min. Extracts were simultaneously concentrated to ca. 2 ml and dialyzed against 1 liter of H_2O for 24 h by sieve filtration under vacuum in a Schleicher & Schuell Co. (Keene, N.H.) collodion bag apparatus equipped with a membrane with a 10,000-molecular-weight cutoff. Dialysis was continued 12 h longer against 1 liter of fresh H_2O, and then 12 h with 1 liter of 50 mM sodium acetate (HCl) buffer (pH 4.0) containing 10 mM KCl and 1 mM each of $CaCl_2$, $MgCl_2$, and $MnCl_2$. The concentrated, dialyzed extracts were diluted to 15 ml with the same buffer-mineral solution. Protein content was assayed by Coomassie blue dye binding (35). Increased absorbance at 595 nm was measured in a 1-cm curette with bovine serum albumin as a standard (1 mg of protein per ml of dye complex gives an optical density of 85.7). Values are reported as mean ± standard deviation for triplicate extracts.

Enzyme assays. Enzyme assays were at 22°C in 50 mM at pH 4.0 (near the in vivo pH [23]) sodium acetate (HCl) buffer. Enzyme (culture extract) was added to initiate the reactions. Where possible, assays were run to 5 to 10% substrate conversion (5 min to 5 h). Extracts with high titers were diluted with the above buffer-mineral solution, and the assays were terminated before reaching either 25% substrate conversion or optical densities of 2.0. Assays were background corrected by the use of zero-time controls. Experimental molar extinction coefficients (ε) for standards are given where possible. Unless otherwise stated, a unit (international unit) of activity was defined as the amount of enzyme required to produce 1 μmol of product per min. Values are reported as units extracted per culture: mean ± standard deviation from triplicate cultures.

Enzymes were assayed by several procedures. Hydrolyases releasing PNP from PNP analogs were assayed by the method of Hägerdal et al. (15) with 3.3 mM substrate. Increased absorbance at 400 nm was measured using PNP as a standard ($\epsilon = 1.83 \times 10^4$). Saccharidases or polysaccharidases releasing reducing sugars from disaccharides or polysaccharides were assayed by the method of Somogyi (34) with a 0.5% solution or suspension of substrate. Increased absorbance at 520 nm was measured by using D-glucose as a standard ($\epsilon = 3.64 \times 10^3$). Cellulase activity was confirmed, and protease was assayed by the method of Rinderknecht et al. (29) with 2.5 mg of substrate (insoluble cellulose azure and hide-powder azure, respectively) per ml. Increased absorbance at 595 nm was measured. A unit of activity was defined as the amount of enzyme necessary to liberate 0.1 optical density unit (1-cm curette) of azure per min. Phenol oxidases were assayed by the method of Leatham and Stahmann (23) with 1.0 mM phenolic substrate (laccase, *o*-tolidine dihydrochloride; phenoloxidase, levodopa; tyrosinase, L-tyrosine). Increased absorbance at 600 nm (laccase) or 460 nm (other oxidases) was measured by using sodium periodate oxidized (23) *o*-tolidine or levodopa as a standard ($\epsilon = 6.34 \times 10^3$ or 3.85×10^3, respectively). Cellobiose:quinone oxidoreductase was assayed by the method of Westermark and Eriksson (43) in 13% (vol/vol) ethanol with 0.8 mM, 3,5-di-*t*-butylbenzoquinone and 1.7 mM cellobiose. Decreased absorbance at 420 nm was measured by using 3,5-di-*t*-butylbenzoquinone as a standard ($\epsilon = 1.54 \times 10^3$).

RESULTS

Dry weight, carbohydrate, and lignin loss. Losses in dry weight, carbohydrates, and lignin during the 150-day cultivation are summarized in Fig. 1 and Table 1. A 25% loss in total dry weight occurred, most of it between days 15 and 90 (Fig. 1). Loss in total carbohydrates followed the weight loss, except between days 45 and 75, when little carbohydrate was degraded. The sum of the weight losses of individual components agreed with the total dry weight loss (Table 1). On a weight basis, lignin loss was nearly equal to that of glucan and was twice that of xylan. Weight loss due to minor carbohydrates was negligible. On a relative rate basis, the rates of loss for glucan and xylan were equivalent, lignin was degraded at nearly twice the rate as glucan (40% loss during the 150-day incubation; Fig. 1), and the minor carbohydrates–galactan, arabinan, and uronans—were degraded even faster (Table 1). The pattern of lignin loss was expected; after an initial lag, lignin degradation was highest early in the incubation.

Vegetative growth pattern and growth-limiting factor. Fungal growth at 22°C was assessed visually and was estimated from changes in culture composition. Inoculum growth into the wood was apparent after 3 days, and by day 15 a thin network of mycelium had permeated the medium. Based on changes in dry weight (Fig. 1), in moisture content (Fig. 2), in glucosamine content (Fig. 3), and in enzyme titers (de-

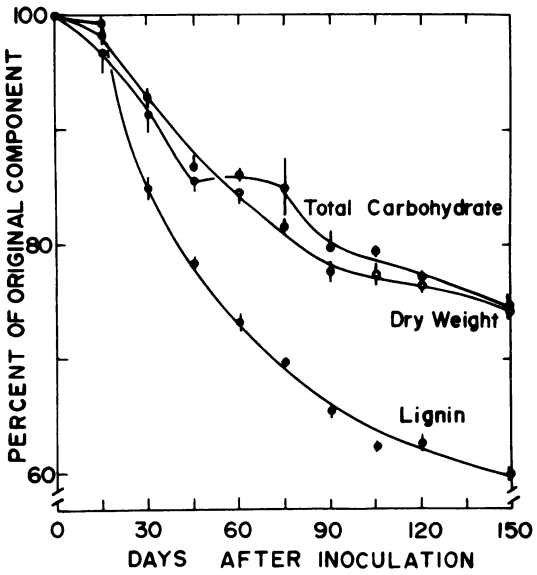

FIG. 1. Dry weight, total carbohydrate content, and lignin content of the cultures during incubation.

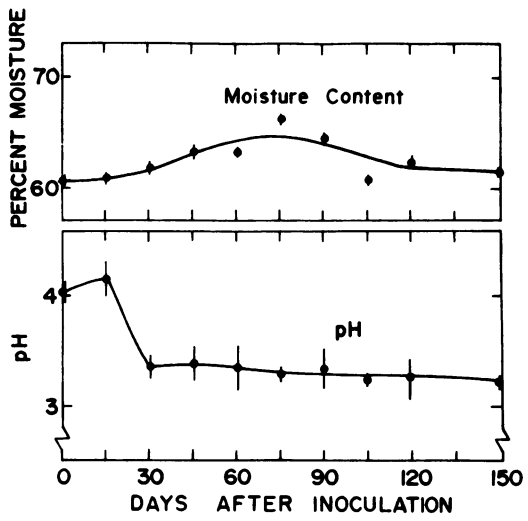

FIG. 2. Moisture content and pH of the cultures during incubation.

scribed below), the most rapid growth occurred between days 15 and 45. The growth rate then slowed. From day 90 onward, decreased dry weight no longer correlated with increased glucosamine content (Fig. 1 and 3). After increasing through day 90 in parallel with growth, the glucosamine content then abruptly increased more than fivefold. Without a cold treatment as stimulus, fruiting did not occur.

Nutrient nitrogen was the probable growth-limiting factor. The medium contained less than 0.5% (dry weight) total nitrogen, at least half of which was from oatmeal protein. Extractable protein rapidly decreased during the incubation until day 60, after which it remained constant (Fig. 3), indicating the depletion of utilizable nitrogen. Neither pH nor moisture changes was growth limiting because neither paralleled the decrease in vegetative growth rate after day 90 (Fig. 1 and 2).

Ligninolytic system activity. Ligninolytic activity ([^{14}C]lignin → $^{14}CO_2$) was maximal in 15-day-old cultures and then decreased sharply with age. In both 24-h and 60-day assays (see Materials and Methods), the pattern of activity paralleled that of lignin loss (Fig. 1 and 4). Interestingly, during the first 1 to 3 days of assay, the rates of lignin degradation were ten-fold lower than the rates thereafter (24-h and 60-day assays, Fig. 4).

TABLE 1. Culture composition and the degradation of specific components by *L. edodes* during the initial 90 days of incubation

Culture component	% of initial dry wt	Relative rate of degradation (% lost/day)	Wt of component lost (g/100 g of medium)
Dry wt	100.0	0.25	22.5
Lignin	22.7	0.38	7.7
Total carbohydrate	63.4	0.22	12.5
Glucan[a]	42.6	0.22	8.5
Xylan[a]	16.4	0.23	3.4
Uronan[a]	1.8	0.35	0.6
Mannan[a]	1.3	–[b]	–[b]
Arabinan[a]	0.8	0.57	0.4
Galactan[a]	0.6	1.17	0.6

[a] Total sugar contents (polymeric, oligomeric, etc.) were measured. Contents are reported as total glucose, xylose, uronic anhydrides, mannose, arabinose, and galactose, respectively, in acid hydrolysates. Values were corrected for destruction during hydrolysis.

[b] Mannan degradation could not be determined in the hydrolysates because a net increase in mannose occurred due to the production of fungal mannan.

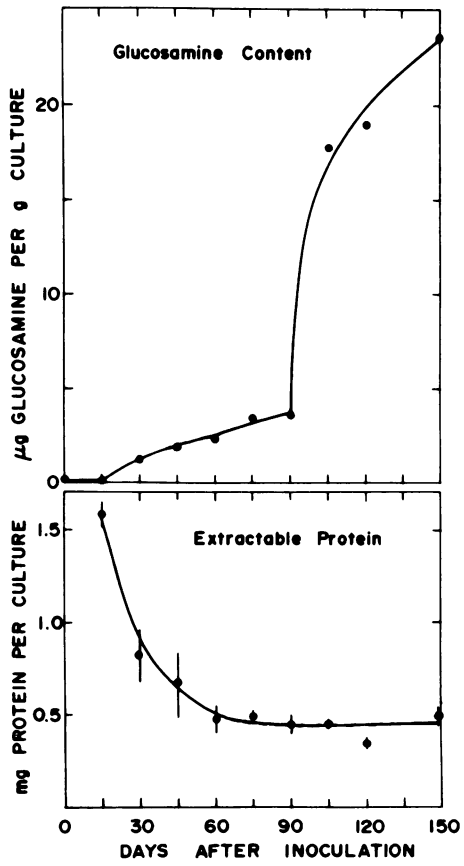

FIG. 3. Glucosamine and extractable protein content in the cultures during incubation.

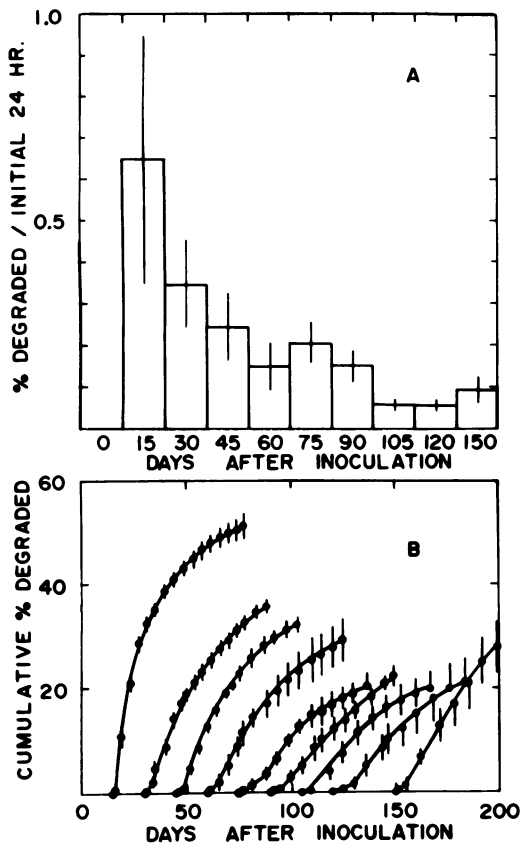

FIG. 4. Relative rates and extents of U-ring-^{14}C-synthetic lignin degradation by culture samples at different ages as determined by a radiorespirometric assay. Values for the initial rates of degradation are expressed as the percentage of labeled substrate degraded to $^{14}CO_2$ in 24 h (A); the extents of degradation during 60-day incubations are expressed as the cumulative percentage of labeled substrate degraded to $^{14}CO_2$ (B).

Extracellular enzymes. The cellulolytic system enzymes were assayed with the appropriate substrates (Fig. 5; not all data shown). Substrates requiring the combined hydrolytic activities of exo- and endo-1,4-D-glucanases for rapid hydrolysis—cotton, solka-floc, cellulose azure, and Avicel—were only slowly hydrolyzed by the extracted enzymes. Substrates for endo-1,4-β-D-glucanase and β-glucosidase—high-viscosity carboxymethylcellulose and salicin, respectively—were hydrolyzed more rapidly. The cellobiose:quinone oxidioreductase substrate DBBQ was either oxidized extremely slowly by the culture extracts or not at all.

Hemicellulases, (gluco)amylase, pectinase, other polysaccharides, and saccharides were assayed with the appropriate polysaccharides and disaccharides (Fig. 5; not all data shown). Major wood hemicelluloses, including glucuronoxylan, arabinoglucuronoxylan, and glucomannan, were readily hydrolyzed by the extracted enzymes. Amylopectin, amylose, polygalacturonic acid, and gum tragacanth (a xylanogalacturonan) were also hydrolyzed at appreciable rates. In contrast, arabinogalactan and locust bean gum (a galactomannan) were only slowly hydrolyzed, whereas gum arabic (a galactoarabinan), gum karaya (a glucuronorhamnogalacturonan), yeast cell wall α-mannan, and sucrose were not hydrolyzed.

Selected classes of hydrolytic enzymes were assayed with PNP analogs, which provide highly sensitive assays. Not all of the PNP sugar analogs were substrates (Fig. 6 and 7; not all data shown). Those that were substrates included PNP-β-D-glucoside, PNP-α-L-arabinoside, PNP-α-D-mannoside, PNP-β-D-mannoside, PNP-α-D-galactoside, PNP-β-D-galactoside, PNP-β-*N*-acetyl-D-glucosaminide, and PNP phosphate ester. The following were either poor substrates or nonsubstrates: PNP-β-D-xyloside, PNP-α-D-xyloside, PNP-α-D-glucoside, PNP-α-D-maltoside, PNP-β-D-glucuronoside, and PNP sulfate ester.

Other enzymes were also detected in the extracts with different substrates (Fig. 7; not all data shown). Acid protease was detected with hide-powder azure, and laccase was detected with *o*-tolidine. Tyrosinase and polyphenol oxidase (active at in vivo pH) were absent at all ages; i.e., the extracts failed to oxidize L-tyrosine and levodopa at pH 4.

Age-dependent changes in enzyme activities. Age (growth)-dependent changes in activities were noted with several enzymes (Fig. 5, 6, and 7). These were enzymes that probably degrade the cell wall polymers of this fungus: β-D-glucosidase, β-D-mannosidase, α-D-galactosidase, laminarinase, and β-*N*-acetyl-D-glucosaminidase. All peaked during the most active phase of fungal growth (days 15

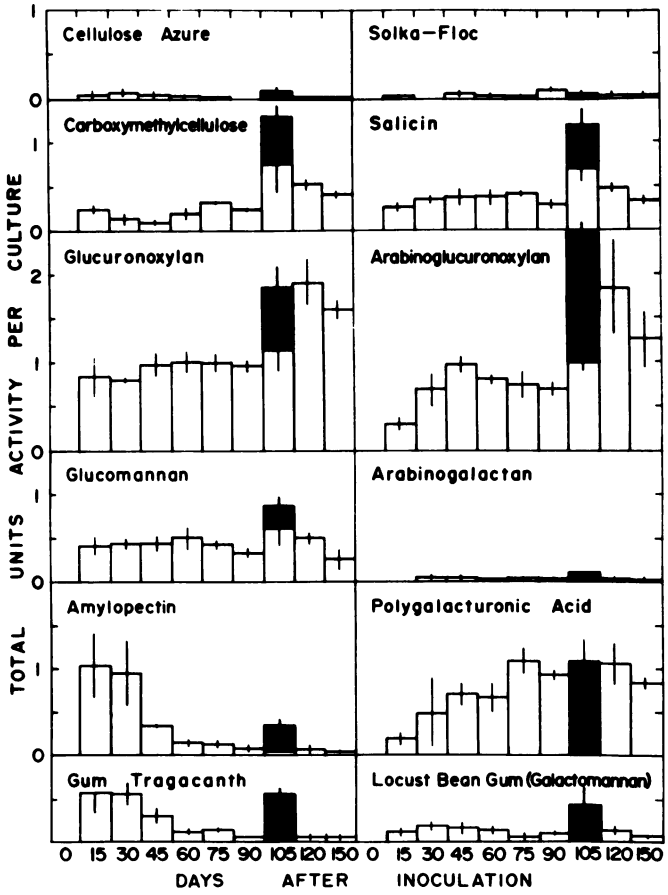

FIG. 5. Degradation of carbohydrates by extractable enzymes from cultures at different ages given (■) or not given (□) a cold treatment (fruiting stimulus) at day 90. Enzyme titers based on individual substances are expressed as total units of activity extracted per culture. Note that in the absence of a cold treatment essentially no activity was detected with cellulose azure, solka floc, arabinogalactan, amylopectin, gum tragacanth, or locust bean gum as the substrate.

through 60) and then again between days 105 and 150. Laminarinase and β-*N*-acetyl-D-glucosaminidase had the most distinct patterns. (Interestingly, laminarinase was also the most active enzyme studied; nearly 4 U per culture was present at day 45.) Peaks in titers also occurred between day 105 and 150 for several other enzymes: endo-1,4-β-D-glucanase, β-D-glucosidase, hemicellulases (acting on glucuronoxylan, arabinoglucuronoxylan, PNP-α-L-arabinoside, and PNP-β-D-galactoside), and acid phosphatase. These peaks in activity did not coincide with significant increases in the rate of medium degradation (Fig. 1).

Other age-dependent changes in the activities were also noted (Fig. 4, 5, and 7). Protease activity, not detected in 15-day-old cultures, was present from day 30 onward, correlating with the loss in extractable protein (Fig. 2 and 7). Despite the low mycelium content of cultures, hemicellulases, (gluco)amylase, and the ligninolytic system were high in young cultures (Fig. 5). The activities of the hemicellulases acting on glucuronoxylan remained essentially constant during the initial 90 days of incubation (Fig. 5). The pattern for laccase activity was complex, but in two separate experiments was clearly age dependent (Fig. 7).

Cold treatment-dependent changes in enzyme titer. Certain extractable enzymes increased in activity 1.2- to 20-fold in 105-day-old cultures which had been subjected to the cold treatment (Fig. 5, 6, and 7). These included laminarinase,

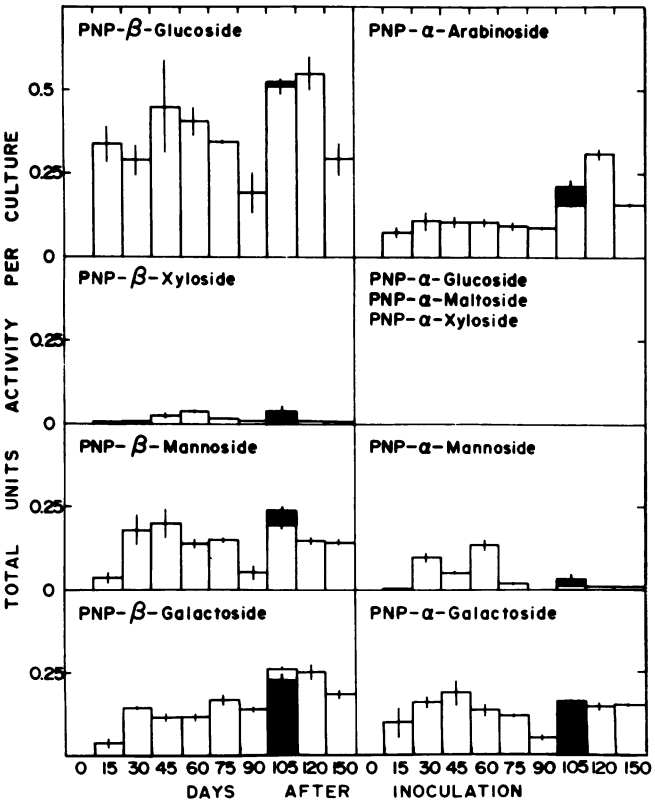

FIG. 6. Degradation of PNP carbohydrate analogs by extractable enzymes from cultures at different ages given (■) or not given (□) a cold treatment (fruiting stimulus) at day 90. Enzyme titers are based on the individual substrates and are expressed as total units of activity extracted per culture. Note that in the absence of a cold treatment essentially no activity was detected with PNP-β-xyloside or PNP-α-mannoside as the substrate.

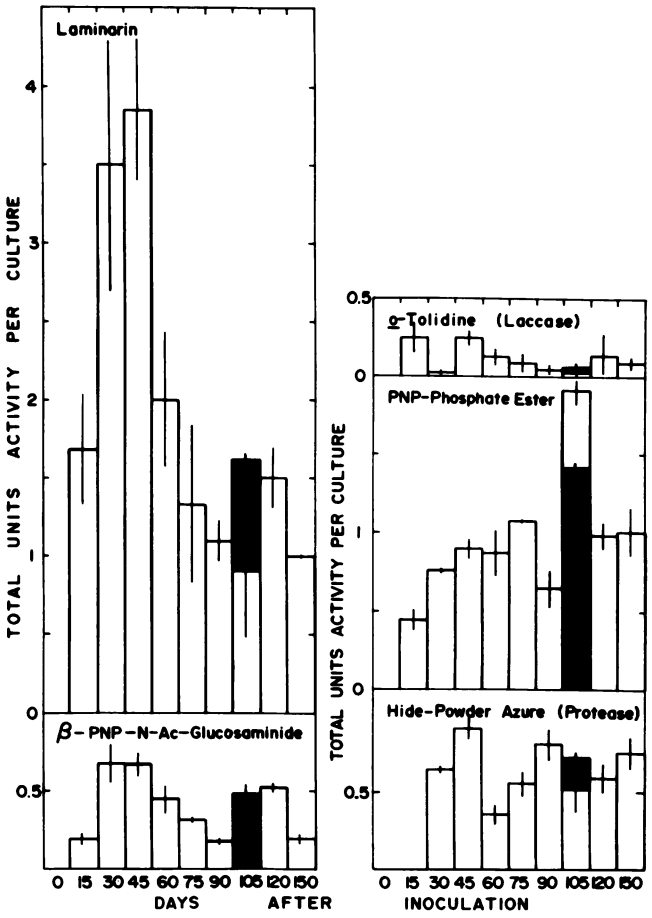

FIG. 7. Degradation or oxidation of substrates by extractable enzymes from cultures at different ages given (■) or not given (□) a cold treatment (fruiting stimulus) at day 90. Enzyme titers are based on individual substrates and are expressed as total units of activity extracted per culture.

endo-1,4-β-D-glucanase, β-D-glucosidase (acting on salicin), hemicellulases (those acting on PNP-β-D-xyloside, PNP-β-D-mannoside, PNP-α-arabinoside, and plant hemicelluloses or gums), (gluco)amylase, acid protease, and laccase. Some of the increases were apparently due to early expression of the enzymes that normally peaked at day 120. However, the β-D-mannosidase, endo-1,4-β-D-glucanase, and β-D-glucosidase activities were higher in cold-treated 105-day-old cultures than in nontreated cultures of any age. Increased (gluco)amylase activity did not occur without the fruiting stimulus. Only β-D-galactosidase (Fig. 6) and acid phosphatase (Fig. 7) significantly decreased due to the treatment.

DISCUSSION

This study has determined the major components degraded by *L. edodes* in a practical oakwood-oatmeal medium and has identified enzymes associated with medium degradation or with growth and development. The assays most useful for the detection and study of each enzyme are summarized in Table 2. Apparent Enzyme Nomenclature Committee (EC) numbers are assigned where possible. The various enzymes are discussed below.

Ligninolytic activity, which is key to wood degradation by white-rot fungi, was present from day 15 onward. As in

TABLE 2. Assay substrates and techniques useful for the detection and study of extracelluar enzymes and the ligninolytic system of *L. edodes*

Probable enzyme (system) degrading natural exracellular substrates[a]	Apparent EC no.[b]	Assay substrate	Basis for assay[c]
Cellulose and its partial degradation products			
Exo-1-4-β-D-glucanase (exo-cellobiohydrolase)	3.2.1.91	Cellulose azure	Azure solubilization
Exo-1-4-β-D-glucanase (exo-cellobiohydrolase)	3.2.1.91	Cellulose (Solka-floc SW-40)	Reducing sugar formation
Endo-1,4-β-D-glucanase (endo-cellulase)	3.2.1.4	Caboxymethylcellulose	Reducing sugar formation
β-D-Glucosidase (cellobiase)	3.2.1.21	Salicin	Reducing sugar formation
Starch, hemicelluloses, and other water-soluble polysaccharides			
(Gluco)amylase	3.2.1(3 or 1)	Amylopectin[d] or amylose	Reducing sugar formation
Hemicellulase(s)[e]	–[e]	Glucuronoxylan (aspen)	Reducing sugar formation
Hemicellulase(s)[f]	–[f]	Arabinoglucuronoxylan (larch)	Reducing sugar formation
Hemicellulase(s)[g]	–[g]	Glucomannan (larch)	Reducing sugar formation
α-L-Arabinosidase	3.2.1.55	PNP-α-L-arabinofuranoside	PNP release
β-D-Xylosidase[h]	3.2.1.(37 or 32)	PNP-β-D-xylopyranoside[h]	PNP release
β-D-Galactosidase	3.2.1.23	PNP-β-D-galactopyranoside	PNP release
α-D-Galactosidase[i]	3.2.1.22	PNP-α-D-galactopyranoside	PNP release
β-D-Mannosidase[i]	3.2.1.25	PNP-β-D-mannopyranoside	PNP release
α-D-Mannosidase	3.2.1.24	PNP-α-D-mannopyranoside	PNP release
Polygalacturonase-pectinase	3.2.1.(67/15)	Poly-1,4-α-D-galacuronic acid–pectin (citrus)	Reducing sugar formation
Lignin			
Ligninolytic system	None assigned	U-ring-^{14}C-synthetic lignin	Radiorespirometric $^{14}CO_2$[c]
Protein			
Acid protease	3.4.99	Hide-powder azure	Azure solubilization
Water-insoluble fungal cell wall polysaccharides and their partial degradation products			
Laminarinase or endo-1,6-β-D-glucanase (or both)	3.2.1.(6, 39, or 75)	Laminarin	Reducing sugar formation
β-D-Glucosidase	3.2.1.21	PNP-β-D-glucoside[j]	PNP release
Chitinase	3.2.1.14	Chitin	Reducing sugar formation
β-*N*-Acetyl-D-glucosaminidase	3.2.1.30	PNP-β-*N*-acetyl-D-glucosaminide	PNP release
Unknown subtrates			
Acid phosphatase	3.1.3.2	PNP-phosphate ester	PNP release
Laccase	1.10.3.2	*o*-Tolidine	Oxidation

[a] Individual enzymes may catalyze single or multiple reactions.
[b] Definitive assignment of EC (Enzyme Nomenclature Committee) numbers requires purification of each enzyme and the determination of substrate specificity.
[c] All assays are at pH 4.0 with H_2O-extracted enzymes, except with the radiorespirometric assays, where the assays were at the in vivo pH with nonextracted culture samples as the ligninolytic system source.
[d] Although amylopectin also requires isoamylase (EC 3.2.1.68) for its complete degradation, it is soluble and thus easier to use than amylose.
[e] Likely to include endo-1,4,β-D-xylanase (EC 3.2.1.8) and 1,2-α-D-glucuronidase (EC number not yet assigned).
[f] Likely to include the enzymes listed in footnote *e* and α-L-arabinosidase (EC 3.2.1.55).
[g] Likely to include endo-1,4-β-D-mannase (EC 3.2.1.78), β-D-mannosidase (EC 3.2.1.25), exo-1,4-β-D-glucosidase (EC 3.2.1.74), endo-1,4-β-D-glucanase (EC 3.2.1.4), and β-D-glucosidase (EC 3.2.1.21).
[h] Although the PNP analog is a poor substrate and its degradation pattern does not predict the patterns for xylan degradation, use of PNP-β-D-xylanpyranoside does detect a β-xylosidase.
[i] May degrade the cell wall L-fuco-D-manno-α-D-galactan in fruit bodies of *L. edodes* or hemicelluloses.
[j] May actually detect one or more 1,6-, 1,3-, or 1,4-β-D-glucosidases or β-D-glucanases.

Phanerochaete chrysosporium, the initial lag in activity at the start of each assay was apparently associated with culture perturbation (20). In agreement with previous results (22), and in contrast to results with the widely studied lignin-degrader *P. chrysosporium* (1, 20), the activity here was associated with neither depletion of culture nitrogen nor with phenoloxidase activity. Ligninolytic activity was maximal here when glucan degradation was maximal, suggesting that *L. edodes*, like *P. chrysosporium* (5, 19), must degrade an alternate substrate to support lignin degradation. *L. edodes* apparently fails to hydrolyze wood polysaccharides at rates sufficient for both optimal growth and maintenance of high ligninolytic activity. Studies in progress now have demonstrated that adding easily metabolized carbohydrates

such as starch, glucose, or xylose to cultures of *L. edodes* stimulates ligninolytic activity.

Like ligninolytic activity, starch-degrading enzyme activity [(gluco)amylase] was high early in the incubation. This probably accounts for the observed pattern of total glucan loss. *L. edodes* is known to be a vigorous starch degrader (9). Glucose released by amylase action here probably suppressed cellulose degradation (27, 47). However, since the total glucan loss only slightly exceeded the total starch present (starch was 14% of total glucan), it is likely that little of the cellulose (less than 6%) was degraded even after the starch was depleted.

In accord with the low extent of cellulose degraded, the data here suggest that *L. edodes* is at best only moderately cellulolytic. Cellulolytic systems of white-rot fungi are comprised of the hydrolytic enzymes exo-1,4-β-D-glucanase (exo-cellobiohydrolase), endo-1,4-β-D-glucanase (endocellulase), and β-D-glucosidase (cellobiase) (47). Here endo-1,4-β-D-glucanase and β-D-glucosidase were present at moderate levels. Although difficult to extract and thus often underestimated, exo-1,4-β-D-glucanase is often present in only low quantities, limiting the rate at which white-rot fungi degrade native cellulose (28, 47). The exceedingly low activity found here suggests that this is also the case with *L. edodes*. Consistent with only low cellulolytic activity, unpublished results show that *L. edodes* grows with great difficulty on native cellulose as a sole carbon source, and observation shows that despite heavy mushroom production, logs colonized by *L. edodes* retain fiber integrity for many years. In certain white-rot fungi the oxidative enzyme cellobiose:quinone oxidoreductase is apparently also involved in cellulose degradation (10, 47); but it is essentially absent in *Pleurotus ostreatus* Fr. (2), another widely cultivated white-rot mushroom, and, as shown here, it is also apparently absent in *L. edodes*.

In contrast to cellulases, some hemicellulases and polygalacturonidase (pectinase) were extracted in high titers from the cultures. The best substrates for these enzymes were polymers with the common glycosidic linkages 1,4-β-D-xylosyl, 1,4-β-D-mannosyl, 1,4-β-D-glucosyl, 1,2-α-D-glucuronosyl acid, 1,3-α-L-arabinosyl, and 1,4-α-D-galacturonosyl (44). As much as 2 U of xylanase per culture was present. If the growth medium had been solely glucuronoxylan, as assayed with glucuronoxylan, this activity would have been sufficient for complete hydrolysis of this dominant hemicellulose in 4 days. The fact that the glucuronoxylan in the medium was degraded at a much lower rate probably reflects masking by other components, although accumulation of inhibitory end products or slow diffusion of the enzymes, substrates, or end products are also possible explanations. Unlike the wood polymers, many of the PNP analogs tested were poor substrates or were not substrates for these enzymes. These analogs may simply be too small to be substrates for at least the polysaccharides present (27).

Poor ability or failure of the extracted enzymes to degrade certain hemicelluloses showed that other hemicellulases were not produced by the fungus, were poorly extracted, or were not induced. For example, larch glucomannan was degraded at less than half the rate of either glucuronoxylan or arabinoglucuronoxylan. Unlike the latter two polysaccharides, it contains 2 to 5% of a less common 1,6-α-D-galactosyl linkage (44). This suggests a low endo-1,6-α-D-galactanase titer. Likewise, based on the poor degradation of other substrates (see linkages) (3), other enzymes low in activity or not detected included endo-1,3-β-D-galactanase, exo-1,2(1,3)-α-D-mannosidase, and β-L-arabinosidase. Failure to detect invertase might be because it was not induced or because it is cell bound (12, 36).

Enzymes that modify fungal cell walls were readily evident here. Laminarinase, β-D-glucosidase, and β-D-mannosidase peaked near day 45 in a pattern correlated with the rate of vegetative growth. These enzymes are essential for fungal growth and development (6, 12, 45). In *L. edodes* the cell wall β-D-glucans (33), chitin (33), and L-fuco-D-manno-α-D-galactan (32) or their partial breakdown products are the probable substrates. Interestingly, the data for these hydrolases suggest that the undetermined mannose linkage in the galactan (32) is β-D-mannosyl rather than α-D-mannosyl. Other enzymes detected here with likely functions in the cell wall region are laccase (11, 23, 24) and acid phosphatase (7, 13). The complex changes in laccase titer suggest that it has more than one function (5).

The striking accumulation of glucosamine (or its oligomers or polymer) late in the incubation probably reflects the onset of secondary metabolism and the associated preparation for fruiting. The fact that the rapid increase occurred with little weight loss shows that it was not simply due to vegetative growth (14, 48). This also shows that increased glucosamine content does not accurately correlate with fungal growth in *L. edodes*. During the life cycle of basidiomycetes, numerous exceptionally thick-walled primordia are formed, only a fraction of which can expand into mushrooms (25, 42). The accumulated glucosamine probably represents cell wall precursors or polymers slated for primordia and mushroom formation. This interpretation is supported by the work of Tokimoto and Fukuda (37), who reported that high glucosamine content in logs colonized by *L. edodes* correlates with high subsequent mushroom yields and that repeated fruiting decreases the content.

Basidiomycetes characteristically fruit while nutrient limited and in response to an environmental stimulus. The strain of *L. edodes* studied generally fruits on the wood medium used here only when incubated at temperatures that are suboptimal for vegetative growth (12 to 18°C) or when given a cold treatment (e.g., 5°C for 3 to 12 days; Leatham, unpublished results). With the appropriate temperature, sporadic and limited fruiting occurs at about 60 days after inoculation; prolonged, more reproducible fruiting occurs 105 to 150 days after inoculation. The data here suggest that these cultures went through two marked changes in nutrient availability, both of which appear to permit fruiting: (i) when the starch is depleted (transient carbon limitation) and (ii) when utilizable protein is depleted (prolonged nitrogen limitation). Consistent with carbohydrate and nitrogen availability being important to development, the addition of simple sugars (e.g., sucrose or glucose) to such media can reduce the time of the first fruiting to 40 to 45 days, whereas the addition of excess nutrient nitrogen prevents the later prolonged fruiting (Robert Harris, personal communication). When grown on a glucose- or starch-containing liquid medium, the fungus fruits at a constant temperature of 22°C in response to nitrogen limitation (23; G. F. Leatham, *in* D. A. Moore, ed., *Developmental Biology of Agarics*, in press).

The relatively late peaking between days 105 and 150 of several of the enzymes studied here appears to mark either readiness to fruit or the initiation of fruiting. Those enzymes include laminarinase, β-*N*-acetyl-D-glucosaminidase, endo-1,4-β-D-glucanase, β-D-glucosidase, some hemicellulases, and acid phosphatase. Supporting a relationship with fruiting is the fact that the cold treatment (fruiting stimulus) caused similar peaking to occur. Previous studies with *L. edodes* on

hardwood logs linked fruiting to increased cellulase and xylanase titers (17, 40). This enzyme increase and those reported here suggest that localized hydrolysis beneath the site of development helps fuel fruit body expansion. Supporting the importance of extracellular carbon source to development, cultures grown in liquid media require an external carbon source to fruit (39; Leatham, in press).

In conclusion, the results presented here provide a body of new information valuable for optimizing substrate composition and for monitoring and controlling the growth and fruiting of *L. edodes*.

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