ISOZYME PATTERNS OF SELECTED ISOLATES OF PHOLIOTA IN THE STIRPS "ADIPOSA"

by

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1. Introduction

The genus Pholiota (Fr.) Kummer has been placed in the Strophariaceae by most recent taxonomists (Singer, 1975; Smith, 1973). The North American species have been monographed twice: by Overholts (1927) and by Smith and Hesler (1968). Although the treatise by Smith and Hesler is extensive, the authors indicate that their work is not complete, but only a more modern approach to the taxonomy of Pholiota. Singer (1975) stated more recently that problems still exist in the taxonomy of the genus at the species level. The early history of the genus has been reviewed by Smith and Hesler (1968).

Overholts (1927) divided Pholiota into sections and groups. Two of the groups, "Adiposa" and "Tuberculosa," are placed in the section "Truncigeni". The species in the "Adiposa" group are delineated by a combination of smooth spores, a scaly and often viscid pileus, a scaly stipe and brown cystidia in the hymenium. In general the species are described as being fleshy, large, and heavy with yellowish colors predominating. Pholiota adiposa (Fr.) Kummer, P. aurivella (Fr.) Kummer, P. aurivelloides Overholts, P. flammans (Fr.) Kummer, P. squarrosa (Müller ex Fr.) Kummer, P. squarrosoides (Pk.) Sacc., and P. rigidipes Pk. are the species included in this group. Contrary to Harper's (1912) classification, Overholts (1927) comments that he wanted to unite the "Adiposa" and the "Squarrosa" groups, even if they could be separated by the differences in the type of scaly covering on the pileus. Overholts put P. limonella (Pk.)

Sacc. in the "Tuberculosa" group, but Harper put this species in the "Adiposa" group. The "Tuberculosa" group, as described by Overholts (1927), includes those species that have smooth spores, a scaly, viscid pileus and a scaly stipe, but no cystidia. He further describes species in this group as small and brightly colored with reds and oranges predominating.

Smith and Hesler (1968) further define the relationships between these closely related taxa by introducing the stirps "Squarrosa," "Squarrosoides", and "Adiposa". Stirps "Squarrosa" contains P. squarrosa and P. kodiakensis Smith and Hesler, whereas P. squarrosoides and P. rigidipes, among others, are included in the stirps "Squarrosoides". P. adiposa, P. aurivella, P. aurivelloides, P. flammans, P. limonella are grouped in "Adiposa" with several other species. Smith and Hesler (1968) describe species in the latter stirps as having a gelatinous pellicle or subcutis and a gelatinizing subhymenium. They indicate, however, that there is some intergradation with characters attributed to the stirps "Squarrosoides." Kühner and Romagnesi (1953) put P. adiposa, P. aurivella, P. flammans, and P. squarrosa in one group, "Squarrosae". It is apparent that there are varying opinions concerning the subgeneric groupings of taxa in Pholiota, because different authors, as indicated above, have placed the species in a variety of arrangements within the genus.

In an attempt to clarify species concept in Pholiota Farr (1974) initiated a study of taxa in the stirps "Adiposa". She studied P. squarrosa in "Squarrosoides", P. aurivella and a group of species in "Adiposa" sensu Smith and Hesler (1968). She studied microscopic

and macroscopic morphology of the fruiting bodies, developed descriptions of cultural characters for each studied taxa, determined mating compatibility between various isolates, and induced fruiting in the laboratory in order to compare wild type characters with those observed in fruiting bodies produced in the laboratory. It was concluded that all studied specimens belonging to stirps "Adiposa" formed one polymorphic taxon and characters traditionally used to separate species could not be used. P. aurivella and P. limonella were incompatible, but false clamp connections were observed in the crosses.

The variability in the morphological characters in the stirps "Adiposa" justifies the seeking of other means to delimit the taxa in this group. Hall (1969) points out that taxonomy based on morphology has many outstanding features but it has also its limitations, since special conditions may be required to produce the necessary diagnostic characters. Modern taxonomy uses morphology but it also uses data collected via biochemistry, serology, molecular biology, genetics, cytology, and developmental morphology (Tyrrell, 1969). Sibley (1962) states that genetic material performs two basic functions: it replicates itself, and it directs the synthesis of protein molecules and hence the sequence of amino acids in a protein. This is a direct translation of the genetic information that determined the sequence; therefore, comparisons between homologous proteins from different species should provide data for classification. Ultimately, the DNA sequence defines the species, but since an investigation of the base parings is not practical for routine taxonomic work, the expression of the genes or the proteins can be taken as an immediate expression of the genetic code.

Electrophoresis characterizes proteins or enzymes by comparing their number, location, and relative mobility by measuring their travel from the point of origin. Some of this information may be helpful in clarifying taxonomic problems in some groups, but not providing aid in other taxa just as one could expect with any character used. The more difficult systematic problems are likely to have an incomplete solution regardless of the method used. It is considered advantageous to use protein characteristics in taxonomy because these molecules are anyway the ultimate product of only one or two cistrons, and therefore, the protein systematist is close to the genotype and can be concerned with the fundamental basis of the phenotype (Garber and Ribbon, 1968).

Representatives from all major fungal groups have been examined by using starch, polyacrylamide, agar, paper, and immunoelectrophoresis (Clare et al., 1968; Franke, 1973; Snider, 1973). The techniques have been adapted by taxonomists studying Fungi Imperfecti, e.g. Fusarium Link ex Fr. (Meyer et al., 1964; Hall, 1967); Verticillium Nees ex Wallr. (Webb et al., 1972) and Humicola Traaen (Moorhouse and de Bertoldi, 1975). Nealson and Garber (1967) studied 32 strains of Aspergillus Mich. ex Fr. belonging to 15 species and nine groups. Starch gel slabs were stained for esterases, leucine aminopeptidases, and phosphatases. The esterase and phosphatase zymograms from these species could be used in defining taxa. Kulik and Brooks (1970) used polyacrylamide gels to check the validity of the Aspergillus speciation proposed by Raper and Fennell (1965). Their taxonomy was based primarily on conidial color and the morphology of the conidial head. Protein

patterns demonstrated by Kulik and Brook supported Raper and Fennell's concept of the taxa in the A. flavus Link group. There was some variability, however, that was probably due to either the testing method or differences in the biochemical processes between taxa. Sorensen et al. (1971) studied A. terreus Thom and A. carneus (v Tiegh.) Blochwitz groups. Protein patterns were obtained by repeated runs of the same sample and they were reproducible. There were, however, considerable differences between strains of the same species and for this reason protein patterns did not appear to be useful diagnostic tool for identification of the Aspergillus species they studied.

Oomycetous fungi, especially plant pathogens, have been subjected to electrophoresis (Clare, 1963; Zentmyer et al., 1974). Polyacrylamide gel electrophoresis has been used to help identify plant pathogens whose cultural and morphological characters do not clearly define them. Zentmyer et al. (1974) used general protein patterns of Phytophthora citricola Sawada and P. cinnamomi Rands to prove that the unknown isolates from avocado were P. citricola.

In the Zygomycetes, Mucor Mich. ex Fr. (Havens 1976) and Thamnidium Link ex Gray (Stout and Shaw, 1973) among others have been used. Havens (1976) tested electrophoresis as a taxonomic tool by staining 31 isolates of Mucor hiemalis Wehmer for various enzymes. He also mated the strains to get genetic evidence for speciation. He discussed reasons for observed dissimilarities in results. He considered the choice of enzymes used, how the results were scores, and the electrophoretic technique itself as the main sources of variation. He concluded, however, that electrophoretic similarity is a useful indicator of taxonomic relatedness. Stout and Shaw (1973)

compared ten different enzyme activities of Thamnidium elegans Link ex Gray and T. anomalum Hesseltine and Anderson in starch gel electrophoresis. Sites of activity for each enzyme varied from none to three in each isolate. Variable sites were observed in some enzymes but usually the migration of the proteins agreed well from sample to sample. The enzyme patterns supported the current separation of the taxa: the latter species being more recently removed to another genus, Ellisomyces Benny and Benjamin (1975).

In Ascomycetes, plant pathogenic and saprophytic genera have been subjected to electrophoresis: Taphrina Fr. (Snider and Kramer, 1974), Ceratocystis Ellis and Halst (Stipes, 1970) Gremmeniella abietina (Lagerb.) Morelet (Dorworth, 1974); Sclerotinia Fuckel (Wong and Willetts, 1973); Neurospora Shear and Dodge (Chang et al. 1962; Reddy and Threlkeld, 1971). Snider and Kramer (1974) investigated 31 species of Taphrina with a total of 91 samples. Results revealed that electrophoresis should not be used alone to determine species because in some cases the interspecific variations were as great as intraspecific variations. It was agreed, though, that electrophoresis was a helpful additional tool to verify species concept. Four taxa of Ceratocystis were used by Stipes (1970) to clarify species concepts in this plant pathogenic genus. Neurospora has commonly been used in electrophoretic analysis. Chang et al. (1962) characterized protein patterns of different species as well as mutant strains of Neurospora. Reddy and Threkeld (1971) used several enzyme patterns in the study of Neurospora: both wild type strains and biochemical and morphological mutants were included in the investigation. Esterase zymograns separated six out of eight Neurospora species in their study.

In Basidiomycetes, parasitic species as well as mycorrhizal and saprophytic taxa have been investigated for their protein patterns. Strains of fourteen species of Ustilago (Pers.) Roussel were analyzed for alpha-esterases, phosphatases, and leucine aminopeptidases by Bradford et al. (1975). Species could not be separated in this way but taxa having graminous hosts and those of having nongraminous hosts could be grouped on the bases of leucine aminopeptidase patterns. Shipton and Fleischmann (1969) found out that the host interferred with some of the Puccinia Pers. species studied. It appears that electrophoretic techniques should be applied with caution to rusts and smuts because of the nature of these fungi as obligate parasites to specific hosts.

Wang and Raper (1970) investigated using polyacrylamide electrophoresis the effects of dikaryozation of the strains of Schizophyllum commune Fr. that differed only by genes controlling sexual morphogenesis. Of fourteen enzyme systems studied, only phenolases with DOPA as substrate were essentially identical in both monokaryons and dikaryons. Schanel et al. (1971) conducted a very thorough study of intracellular and extracellular enzymes in the basidiomycete species Trametes versicolor L. ex Fr. and T. hirsuta Wulfein ex Fr. The main purpose of this study was to compare intra- and extracellular enzymes and general properties of the latter that enabled them to act "in vitro" under different conditions. The authors commented that variations in the behavior of the enzymes should be used as taxonomic criteria, not Rf values. This approach could explain some of the contradictory results in the use of enzyme patterns in taxonomy.

Madhosingh (1970) used extracts of six agaric species to study their tyrosinase patterns. He concluded that the polyacrylamide gel electrophoresis technique is a significant tool in demonstrating the separation of a number of proteins and tyrosinase isoenzymes from the preparation of a number of proteins and tyrosinase isoenzymes from the preparation of each studied species. The technique itself, however, was of limited value in comparing protein patterns obtained from heterogenous preparations of different species.

Twenty-two isolates of Polyporous (Mich.) Fr. ex Fr. representing nine species were subjected to starch gel electrophoresis by Shannon et al. (1973). Several enzyme patterns were used to detect differences between isolates. It was possible to demonstrate differences between species, but investigators called for studies of more enzymes, more isolates, and more species before taxonomic relationships could be established. Kalab and Matlocha (1966) found characteristic protein patterns in 31 species of mushrooms, but the extracts could not be stored without loosing reproducibility.

In his discussion of the use of enzymes in the taxonomy of Basidiomycetes, Blaich (1977) reported that tests for specific enzymes were more sensitive and less time consuming that those of general proteins. Blaich also considered enzymes less influenced by mutations than general proteins. He found out that esterases, aminopeptidases, and phenoloxidases were best suited for taxonomic purposes. Enzymes commonly appear in different forms that dispite their dissimilar molecular entities catalyze one and the same reaction. These different forms of enzymes are called isozymes collectively if they are result of

activity in different gene loci and allozymes if they are coded by one gene locus. As reviewed by Esser and Kuenen (1967) fungal laccases and tyrosinases have several gene loci that code for them. Esterases and peroxidases are also formed by activity of several genes (Gottlieb, 1977), so it is expected that the material under investigation here would display several areas of enzyme activity when crude extract is electrophoresed and stained for an enzyme. Electrophoresis is commonly used for study of heterogenity and multiple forms of proteins, even if single type of electrophoresis with one buffer system and one gel concentration does not really characterize the studied protein (Ressler, 1973). Blaich (1977) points out, though, that when protein characteristics are used as an aid in taxonomy, we should not be concerned about the function of the enzyme, but rather use it solely as a reproducible character, among other characters, in classification.

It is apparent from above examples that electrophoresis is an established tool in the fungal taxonomy, but experiments should be planned very carefully to minimize variations that exist in performing the tests. Electrophoresis has been used most often in cases where there is a shortage of good, morphological characters or when the features used are extremely variable. In order to clarify species concept in Pholiota, stirps "Adiposa", selected strains of P. limonella sensu Farr et al. (1977) and P. aurivella were subjected to electrophoresis:

1. Optimum temperature for growth was established by conducting linear growth studies of the strains under investigation.

2. Dry weight accumulation and respiration studies were carried out from each isolate to enable harvesting of the mycelium at physiologically comparable stages.
3. Soluble proteins were extracted from the mycelium growth in liquid shake culture in order to form:
   a) general protein patterns of soluble proteins,
   b) esterase zymograms, alpha-naphthylacetate as substrate,
   c) phenoloxidase zymograms, DOPA and paraphenylene-diamine as substrate,
   d) peroxidase zymograms, catechol and pyrogallol with hydrogen peroxide as substrate.

2. Material and Methods

2.1 Cultures

The following dikaryotic cultures, except VT 552, Pholiota aurivella, have previously been examined by Farr (1974) for microscopic characters and mating compatibility, and they have been induced to fruit in the laboratory.

Pholiota aurivella (Fr.) Kummer

VT 398, OKM 3028, basidiocarps on scar on living Sorbus americana Marsh., Moscow, Idaho, coll. O. K. Miller, Jr., 11-9-64. (Farr, 1974, p. 20)

VT 552, OKM 7060-Sp., on live hemlock tree trunk, 6 ft. off ground, Beltsville, Md., coll. L. Stewart, 10-20-68.

Pholiota sp. (Fr.) Kummer

VT 394, OKM 2792, basidiocarps on Tsuga stump, Priest River Experimental Forest, Priest River, Idaho, A. H. Smith, 9-23-64. (Farr, 1974, p. 28)

VT 395, OKM 3764, basidiocarps in hollow [illegible] Liquidambar styraciflua L., Laurel, Md., coll. [illegible] Lombard, 11-19-65. (Farr, 1974, p. 28)

VT 397, OKM 4958, basidiocarps on hardwood, Laurel, Md., coll. Mrs. J. Linsay, 10-18-66. (Farr, 1974, p. 28)

VT 403, CS-66-109-12-A, rot isolate from white pocket rot on Carya sp., Shawnee Natl. Forest, Pope Co., Ill., F. H. Berry 12-5-72. (Farr, 1974, p. 29)

VT 450, GAL 550, basidiocarps on wood, Mountain Lake, Giles Co., Va., coll., G & B. Laursen, 10-29-72. (Farr, 1974, p. 28)

VT 451, DF 441, basidiocarps on hardwood log, Grotto's Fall trail, Great Smoky Mountains National Park, Tenn., coll. I. Baxter, 11-11-72. (Farr, 1974, p. 28)

2.2 Culture medium

All fungi were grown on a modified Vogel's (1956) medium as described: 125 g Na-citrate · 2 H_2O; 250 g KH_2PO_4 (anhydr.); 100 g NH_4NO_3 (anhydr.); 10 g $MgSO_4$ · 7 H_2O; 5 g $CaCl_2$; 2 H_2O; 750 ml distilled water. Dextrose was used as carbon source (Lilly and Barnett, 1958; Wongsthientong, 1967). The medium was supplemented with an aqueous solution of yeast extract and thiamine-HCl. Vogel's solution, yeast extract, and thiamine solution were sterilized by membrance filtration (Gelman Metricel GA-8, 0.2 μ pore size). The final culture medium consisted of 20 ml of Vogel's solution, 30 g dextrose, 10 mg yeast extract, 5 mg thiamine-HCl, 1 l distilled water. When ammonium nitrate was replaced with sodium tartrate (4.6 g/l) in the final culture medium, 18 ml Vogel's salt solution was used, other ingredients remaining the same.

2.3 Linear growth studies

The linear growth studies were done in 15x90 mm plastic petri dishes on Vogel's agar (1.5%) medium at 10, 15, 20, and 25 C in non-illuminated constant temperature (± 0.5 C) incubators. The plates were inoculated with actively growing mycelium taken from the edge of the colony with no. 3 cork borer (7 mm inside diameter). The inoculation plug was placed close to the edge of the plate and a line was drawn along the diameter of the plate. Measurements were taken along this line, starting from the edge of the inoculation plug, one observation each time. The growth was measured every three days in four replicate plates. The plates were taped together with masking

tape, and wrapped in aluminum foil to avoid contamination, desiccation, and accidental exposure to light.

2.4 Dry weight determination

The starter colonies were successively grown on plates of Vogel's medium at 25 C. Each starter flask (125 ml Erlenmeyer containing 25 ml of Vogel's broth medium) was inoculated with three agar plugs (no. 1 cork borer, 4 mm inside diameter) taken from the growing colony margin of a starter plate. The flasks were then plugged with cotton and covered with aluminum foil. Incubation was carried out for six days at 25 C without agitation.

The production of mycelium by each isolate varied considerably and subsequently their dry weight production differed from each other. The following was done to standardize the inoculum size, because the size of the inoculum has been found to affect results (Ward and Colotelo, 1960). Four starter flasks from each isolate were harvested for dry weight determination. Each flask had three starter plugs (no. 1) grown for six days. The agar plugs with the mycelium were used for the dry weight determination. From the results it was calculated how many plugs from starter flasks were needed to make homogenate containing 2 mg dry wt/ml when the minced starter plugs were suspended in 40 ml of salt solution (autoclave 18 ml Vogel's solution in 1 l distilled water). To make the homogenate for the inoculation the incubated plugs were transferred to a Waring blender and minced for 10 s at the highest speed. The mycelium was separated from the liquid by centrifugation for 10 min at 12 000 x g (avg) in

Sorvall SS-3 centrifuge. The supernatant was poured out and the mycelial pellet was resuspended in 25 ml of Vogel's salt solution using Vortex mixer. The mycelium was washed once in this way. Then it was rehomogenized for 10 s in a Waring blender. A 1 ml aliquot was used for all inoculations into 50 ml culture medium in 250 ml flasks.

All cultures were grown on an Eberbach reciprocating shaker, 90 cycles/min at 25 C. Triplicate total culture harvest were made every three days for fifteen days. Predied and weighed filter papers were used to collect the mycelium in Buchner funnels. Suction from a water aspirator was used to separate the mycelium from the culture medium. Glass fiber filter papers were used in dry weight determinations because it was determined that their weight stayed most constant during drying. When mycelium was collected for protein extraction regular Whatman no. 1 filter papers were used because the wet strength of this paper was better than that of glass fiber filter papers. Mycelium that was collected on the filter paper was washed twice with ice cold distilled water to free the mycelium from the culture medium. The washed mycelium was scraped off from filter paper with a plastic spatula, the wet weight was recorded, and then the mycelium was frozen. The dry weight determinations were made after the samples had been dried in an oven at 105 C for at least eight hours and cooled in a desiccator. Balance desiccators were used during summer months to control humidity.

2.5 Respiration

Respiration data as µl O_2/h/mg dry weight were collected using Gilson differential respirometers with the direct alkaline absorptiom technique (Umbreit et al., 1964). Four samples were taken six, nine, twelve, and fifteen days after inoculation of liquid cultures as total culture harvest. After transferring the mycelium to reaction flasks the flasks were allowed to equilibrate for two hours at the running temperature, 25 C. Readings were then taken every 30 minutes for two hours. After each run the dry weights of the mycelial pellets from each reaction flask were taken as described.

2.6 Extraction of soluble proteins

The harvested mycelium was kept frozen until used. Extraction of the soluble proteins was carried out in 25 mm Tris-HCl buffer, pH 8.1, containing 0.5 M NaCl, at 2 C. The mycelium was homogenized at 2 C with Brinkman Polytron (probe 20) in 2-5 ml buffer (Steward and Barber, 1964) with three 15 s periods at full speed, 60 s intervals were maintained between extractions. The resulting homogenates were centrifuged for 20 minutes at 13 000 x g (avg) in a Sorvall RC2-B Centrifuge. The supernatant was tested for protein content (Lowry et al., 1951) using bovine serum albumine as standard. The extractions were stored at -4 C until used for general protein and isozyme studies.

2.7 Polyacrylamide gel electrophoresis

Basic, ph 9.5, gels were prepared and run by the procedures of Davis (1964) and Steward and Barber (1964). The running gel was 7%

standard acrylamide-0.18% N,N' methylenebisacrylamide and the stacking gel was 3.125% acrylamide-bisacrylamide (Anon. 1968). The running gels, 90 x 5 mm, were cast and polymerized for one hour at room temperature and 10 mm of stacking gel was then polymerized for one hour at room temperature under fluorescent lights. The gels were allowed to set a 4 C overnight for the use the following day.

Gels were prerun for 15 minutes at 1 mA/tube. Protein samples 40 or 80 µg/gel, were mixed with 0.05% Bromophenol blue (10% v/v) and with sucrose added to a 15% concentration. The samples were layered on the stacking gel and run for 10 minutes at 1 mA/tube. When samples were in the gels the current was raised to 2.5 mA/tube. Gels were run at constant current until the tracking dye reached 90 mm down the gel.

2.8 Staining for general proteins

The samples were stained for general protein patterns in 0.1% Coomassie Brilliant Blue R 250 in 10% acetic acid three hours to overnight modified from Crambach et al., 1967. The gels were destained in 10% acetic acid and stored in acidic distilled water.

2.9 Isozyme stains

2.9.1 Laccase

Five mM paraphenylenediamine was prepared in 0.1 M phosphate buffer, pH 7.4. The gels were incubated in the solution at 40 C until the bands appeared (Blaich, 1977).

2.9.2 Tyrosinase

The gels were incubated for one hour in a 0.1% solution of DL-dihydroxyphenylalanine = DOPA in 0.01 phosphate buffer, pH 7.4. Excessive darkening of the gels was prevented by incubating them in 0.05% $CuSO_4$ aqueous solution for a maximum of 30 minutes (Madhosingh 1970).

2.9.3 Alpha-esterases

Alpha-esterases were stained according to Webb et al. (1972), modified as follows: phosphate buffer was 0.1 M, pH 7.4, with Fast Red TR salt as the dye coupler.

2.9.4 Peroxidases

The gels were immersed in a 20 mM solution of catechol containing 0.3% hydrogenperoxide solution (Rudolph and Stahmann, 1964) or successively flooded with aqueous 1% pyrogallol and 0.3% hydrogenperoxide (Hall, 1967).

2.10 Photography

The general protein patterns and zymogram patterns were photographed with the gels in waterfilled test tubes. Kodak Ektachrome ET-135 was used for recording as well as Kodak Plus X, both films used without filters.

3. Results

3.1 Linear growth studies

Linear growth studies were carried out at 2, 10, 15, 20, 25, 30 C in order to establish the optimum growth temperature for the experiments. The two degree growth studies did not come out of lag phase during the course of the study, and the preliminary studies at 30 C did not notably increase the growth rate, so those temperatures were omitted in the final experiment. For all of the strains 25 C gave the best linear growth. The eight investigated strains fell into three distinct groups. Pholiota limonella, VT 394 was the slowest grower, reaching 35.5 mm in 27 days, an average of 1.31 mm per day. P. limonella (VT 397) and P. aurivella (VT 398) grew at the same rate, 1.80 mm per day. The third group included the rest of the isolates: VT 395 (P. limonella) grew 2.37 mm per day, VT 403 (P. limonella) 2.61 mm per day, VT 451 (P. limonella) and P. aurivella (VT 552) grew both at the rate of 2.69, and the fastest of all isolates was VT 450 (P. limonella) by 2.93 mm per day. The results of the studies that lasted for 27 days at what time the fastest isolate had reached the other edge of the petri plate are reported in tables 1-4, pages 19-22.

3.2 Dry weight and respiration studies

The purpose of the dry weight determinations and respiration studies was to find out comparable harvesting times for each isolate. It is widely accepted and often shown that organisms at different growth phases display activities that cannot be traced at other stages

Table 1

Linear Growth at 10 C

	VT 394	VT 395	VT 397	VT 398	VT 403	VT 450	VT 451	VT 552
3d x	0	0	0	1.0	0	0	0	0
s				0.0				
6d x	1.0	1.8	0	1.3	2.0	2.0	1.0	1.8
s	0.0	0.50		0.50	0.0	0.0	0.0	0.50
9d x	2.0	3.8	1.5	2.7	3.5	3.0	2.8	3.5
s	0.0	0.50	0.58	0.50	0.58	0.0	0.50	0.58
12d x	3.0	4.8	3.0	3.8	5.5	4.5	4.0	5.5
s	0.82	0.50	0.82	1.3	0.58	0.58	0.0	1.0
15d x	5.0	6.0	4.3	4.8	7.3	6.5	5.0	6.8
s	0.0	0.82	1.7	0.50	0.50	0.58	0.0	0.50
18d x	6.5	7.8	5.3	5.5	8.5	8.0	6.8	8.5
s	0.58	0.50	1.7	1.3	0.58	0.82	0.50	0.58
21d x	8.0	9.5	6.3	7.0	10.0	9.5	8.5	9.8
s	0.0	0.58	2.4	1.2	0.0	0.58	0.58	1.5
24d x	10.0	10.8	8.0	8.0	11.8	11.3	10.0	11.5
s	0.0	0.50	2.2	1.4	0.50	0.96	0.0	0.58
27d x	11.5	12.8	10.3	9.5	13.3	13.3	12.0	13.8
s	0.58	0.50	3.1	1.3	0.50	0.50	0.82	0.50

x = mean of four replicates in mm
s = standard deviation
d = days of growth

Table 2

Linear Growth at 15 C

	VT 394	VT 395	VT 397	VT 398	VT 403	VT 450	VT 451	VT 552
3d x	0	1.5	1.3	1.0	1.5	1.0	1.0	1.0
s		0.58	0.50	0.0	0.58	0.0	0.0	0.0
6d x	2.0	4.3	4.0	1.8	5.0	3.3	3.0	4.5
s	0.0	0.50	0.82	0.50	1.5	0.50	0.0	0.58
9d x	4.5	8.0	7.5	3.8	8.5	7.5	6.0*	7.5
s	0.58	0.0	0.50	0.50	0.58	1.3		0.58
12d x	7.5	11.5	10.0	7.3	12.2	10.7	9.5	11.5
s	1.0	1.0	1.8	0.96	1.2	1.5	0.58	0.58
15d x	9.5	15.8	14.3	10.0	15.8	14.0	13.0	14.8
s	0.58	0.50	2.0	0.0	1.3	0.82	0.0	0.50
18d x	12.0	19.8	17.0	12.0	19.3	18.3	16.8	18.3
s	0.82	0.96	1.7	0.0	1.3	1.2	0.50	0.50
21d x	13.8	23.5	20.3	14.5	22.5	21.0	20.3	21.0
s	0.50	0.58	2.0	0.58	1.3	2.6	0.96	0.0
24d x	15.8	26.8	24.7	17.8	26.3	25.0	23.3	24.0
s	1.3	0.50	3.2	0.50	1.3	2.7	1.5	0.82
27d x	17.5	30.5	28.0	20.0	30.0	29.5	28.5	27.8
s	1.0	1.3	2.6	0.58	1.4	1.4	1.0	0.50

x = mean of four replicates in mm *estimated
s = standard deviation
d = days of growth

Table 3

Linear Growth at 20 C

	VT 394	VT 395	VT 397	VT 398	VT 403	VT 450	VT 451	VT 552
3d x	1.0	1.3	1.3	2.3	2.0	2.0	2.3	2.5
s	0.0	0.50	0.50	0.50	0.0	0.0	0.50	0.58
6d x	2.5	6.3	4.5	5.0	7.8	7.0	6.3	7.8
s	1.3	0.95	0.58	0.0	0.50	0.82	0.50	0.96
9d x	6.3	13.3	9.8	9.0	13.0	13.0	10.5	13.3
s	2.6	1.3	0.96	0.0	0.81	0.0	1.7	0.96
12d x	10.0	18.3	16.0	13.3	19.5	19.5	16.0	20.8
s	4.1	0.96	1.1	0.58	0.58	0.58	2.7	1.5
15d x	18.3	25.8	20.8	17.3	26.3	27.5	22.5	25.0
s	3.2	1.3	1.5	0.58	0.50	0.58	3.1	2.6
18d x	20.8	31.5	26.0	22.0	31.3	33.2	28.7	31.0
s	3.9	1.0	1.8	1.0	1.26	1.5	3.6	2.8
21d x	23.3	38.5	32.5	26.7	37.8	42.3	35.3	37.0
s	6.0	1.0	1.7	0.58	0.96	1.0	4.0	2.3
24d x	25.7	45.0	37.3	31.3	43.5	49.0	42.3	43.3
s	6.6	1.4	2.0	0.58	1.0	1.1	4.7	2.4
27d x	31.5	52.3	44.3	35.7	50.0	57.5	47.5	48.8
s	6.5	0.96	2.0	1.1	1.4	1.3	5.9	2.4

x = mean of four replicates in mm
s = standard deviation
d = days of growth

Table 4

Linear Growth at 25 C

	VT 394	VT 395	VT 397	VT 398	VT 403	VT 450	VT 451	VT 552
3d x	1.0	3.0	3.5	2.0	5.0	4.3	2.0	4.8
s	0.1	0.0	0.58	0.0	0.0	0.5	0.0	0.5
6d x	2.5	9.3	9.5	5.5	11.5	12.3	7.5	11.0
s	1.0	0.50	0.58	0.58	0.58	0.50	1.3	2.7
9d x	8.0	16.0	14.0	11.5	18.5	20.8	16.3	20.5
s	3.4	1.4	0.82	1.3	0.96	0.5	0.96	0.58
12d x	13.0	23.5	20.0	17.3	28.3	32.3	25.5	30.3
s	3.2	1.7	1.6	0.5	1.0	0.96	1.7	0.50
15d x	18.5	31.8	24.3	22.8	36.5	41.8	32.3	38.0
s	3.1	1.2	1.7	0.50	2.4	0.96	1.9	0.82
18d x	22.3	40.0	28.3	28.5	44.5	51.8	41.5	46.3
s	4.7	2.2	2.0	0.58	2.4	0.96	3.7	0.50
21d x	27.3	50.5	38.0	35.5	56.8	65.0	54.3	58.5
s	5.3	3.0	2.0	1.3	2.9	1.0	1.5	0.58
24d x	30.5	56.0	41.0	41.0	62.	70.3	63.0	64.2
s	4.4	4.0	2.2	0.82	3.3	2.1	1.8	0.5
27d x	35.5	64.0	48.5	48.5	70.5	*	72.8	72.0
s	1.9	4.7	3.3	1.9	3.1		2.2	0.82

x = mean of four replicates in mm
s = standard deviation
d = days of growth

*growth reached the other edge of the plate

of life cycle (Wang and Raper, 1970; Chet et al. 1972; Lloyd et al., 1972; Chang and Chan, 1973; Brody, 1977). The isolates were harvested either on ninth or twelfth day of growth, depending on the combined data obtained from the dry weight accumulation and oxygen uptake. The maximum respiration was reached before the peak of dry weight accumulation in six out of eight isolates used here (see Figures 1-8, pages 28-35). The two isolates that did not follow the patterns were VT 395 and VT 403, both P. limonella-isolates that grew very slowly.

The slowest grower in liquid culture was VT 395, Pholiota limonella, that reached maximum growth in six days, 28.9 mg (Table 5, page 26). This slow growth caused some difficultires in obtaining enough mycelium for harvesting. Maximum respiration was recorded on the twelfth day, 7.17 µl 0_2/h/mg dry weight (Table 6, page 27, Figure 2, page 29). Pholiota limonella, VT 403, reached the upper part of logarithmic growth phase rather early, 33.9 mg dry weight (Figure 5, page 32), in nine days and respiration was most active on the twelfth day of growth, 7.75 µl 0_2/h/mg dry weight. Pholiota limonella, VT 450, accumulated dry weight slowly but steadily and was still increasing on the fifteenth day, 80.47 mg dry weight. Oxygen uptake of this isolate was highest on the ninth day (Figure 6, page 33). The next isolates, if one looks at the dry weight production on the twelfth day, were VT 398, P. aurivella 64.2 mg dry weight (Figure 4, page 31), and VT 451, P. limonella, 66.55 mg dry weight (Figure 7, page 34). Respiration differed between these two isolates: VT 398 oxygen uptake was 7.67 µl 0_2h/mg dry weight on the ninth day, wherease the maximum in VT 451 was on the twelfth day, 5.76 µl 0_2/h/mg dry weight, Pholiota limonella VT 394, reached maximum respiration in nine days, 5.92 µl 0_2/h/mg dry

weight and produced 81.75 mg dry weight in twelve days (Figure 1, page 28). Pholiota limonella, VT 397, accumulated dry weight steadily all through the experiment, reaching 116.9 mg dry weight in fifteen days, but maximum respiration was observed on the ninth day, 4.73 µl O_2/h/mg dry weight (Figure 3, page 30). Pholiota aurivella, VT 552, formed a distinct group, producing 237.15 mg dry weight in fifteen days. This extremely fast grower was most active in oxygen uptake already on the sixth day of growth, which was the first day of respiration studies (Figure 8, page 35).

The results of the dry weight and respiration studies are reported in Tables 5 and 6, pages 26and 27 and combined oxygen uptake and dry weight accumulation graphs are on pages 28-35.

3.3 General protein patterns

Crude protein extracts were subjected to repeated electrophoresis runs in order to establish reproducible banding patterns on polyacrylamide gels. In one run, different isolates produced from thirteen to twenty-two protein bands that were distinguishable by eye on light table. In another run the number of bands varied from ten to sixteen in different isolates so there was variation from run to run that was partly due to the untrained eye that gradually was schooled to track the protein bands. There was considerable similarity from one run to another that was considered constant enough to proceed further to the isozyme stains (Figure 9, page 36). General protein patterns of all eight isolates are pictured in Figure 10, page 37. There are two strong bands that are common to most of the isolates, around 15-20 (Rf) and another 35-40. These bands (Figure 11, page 38) are more obvious, if the samples

have been treated with extraction-buffer saturated butanol: One part sample and four parts of butanol are mixed and let to stand on ice for 10 minutes. After that the samples are centrifuged for 10 minutes at 3000 x g (avg) and the bottom fraction of the sample is used for electrophoresis. There is nothing in the banding patterns that would clearly distinguish the two species used here, Pholiota aurivella (d and h in Figures 10 and 11) and P. limonella (a, b, c, e, f, g, in Figures 10 and 11).

3.4 Phenoloxidases

In this study laccases have been defined as those compounds that react when p-phenylenediamine is used as substrate (p-diphenol-O_2 oxidoreductases, E.C. 1.14.18.1), whereas tyrosinases have been defined as those enzymes showing DL-DOPA (dihydroxyphenylalanine) activity at pH 7.4 in 0.1 M phosphate buffer (Blaich and Esser, 1975). Phenoloxidases, tyrosinases and laccases, have been of interest to mycologists for their participation in melanin production. Smith and Berry (1974) reported several studies in which the role of melanin in the formation of perithecia has been studied. The fungi investigated include Neurospora crassa, Glomerella cingulata, Podospora anserina, and Hypomyces solani. Phenoloxidases are also involved in sclerotium formation in Sclerotium rolfsii (Chet et al., 1972).

The production of phenoloxidases by wood rotting fungi has been known for some time even if their role in fungi is not well understood. White rot fungi produce tyrosinases and laccases, whereas brown rot fungi produce tyrosinases only, so they appear to have a role in lignin degradation (Blaich and Esser, 1975). Molitoris (1977) studied over

Table 5

Dry Weight Accumulation in mg at 25 C

	VT 394	VT 395	VT 397	VT 398	VT 403	VT 450	VT 451	VT 552
3d x	14.0	12.7	5.1	11.5	8.5	8.5	8.2	10.1
s	0.28	3.5	0.39	2.0	0.42	0.33	2.25	0.42
6d x	27.8	28.9	38.9	20.0	30.3	15.1	12.5	45.45
s	8.56	0.42	0.71	0.28	2.0	3.8	0.64	4.6
9d x	49.6	13.9	66.2	28.6	33.8	23.25	16.75	124.9
s	5.2	1.5	8.4	2.4	7.4	6.6	4.3	18.0
12d x	81.75	12.6	83.6	64.2	34.2	46.4	66.55	194.15
s	5.8	0.71	4.5	17.0	2.1	17.1	11.3	60.4
15d x	82.25	19.0	116.9	57.7	32.35	80.47	85.45	237.05
s	0.07	0.28	5.2	5.8	0.49	6.2	1.4	26.0

x = mean of duplicate samples
s = standard deviation
d = days of growth

Table 6

Respiration at 25 C

		VT 394	VT 395	VT 397	VT 398	VT 403	VT 450	VT 451	VT 552
6d	x	2.23	1.76	3.59	3.91	3.78	2.2*	1.63	6.33
	s	0.98	0.40	0.14	0.23	0.69		0.60	0.34
9d	x	5.92	4.07	4.73	7.67	5.61	8.28*	4.6	3.44
	s	2.4	0.74	0.52	1.2	1.0		2.1	0.23
12d	x	4.36	7.17	3.86	3.68	7.75	7.18	5.76	3.64*
	s	0.25	1.3	1.7	0.31	4.0	1.0	0.29	
15d	x	3.74	4.06	3.58	4.89	1.72	5.19	2.57	3.57
	s	0.47	0.74	0.07	0.22	0.13	0.20	0.01	0.96

x = mean of duplicate samples
s = standard deviation
d = days of growth

*malfunctioning of a manometer, single sample

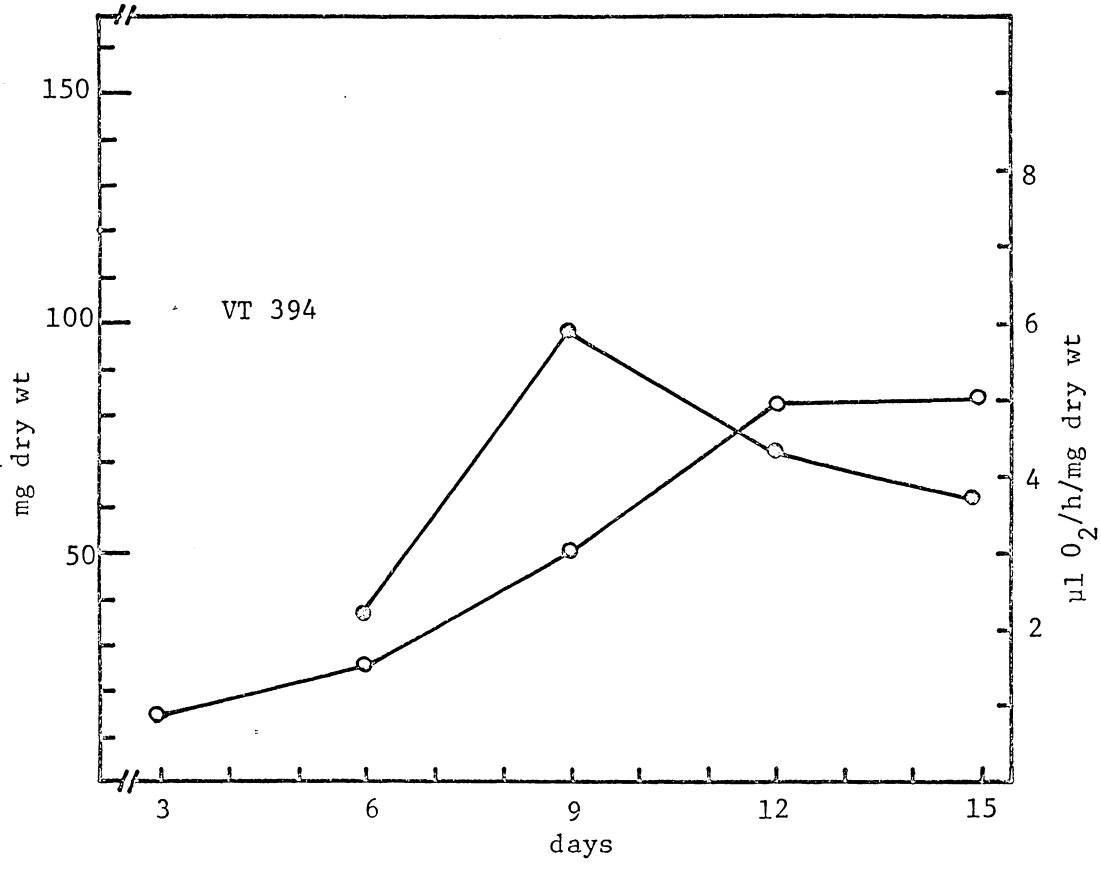

Figure 1. Combined dry weight and oxygen uptake data from Pholiota limonella (VT 394)

● = oxygen uptake
○ = dry weight

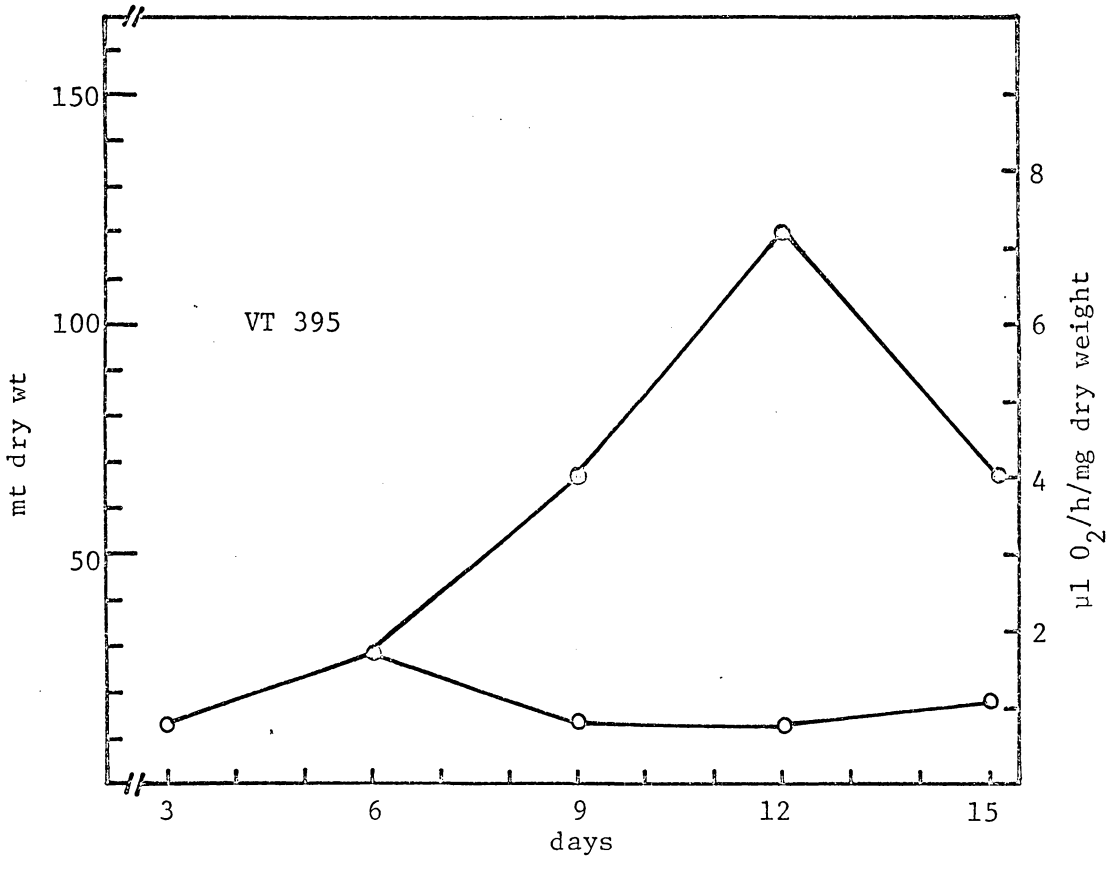

Figure 2. Combined dry weight and oxygen uptake data from Pholiota limonella (VT 395)

● = oxygen uptake
○ = dry weight

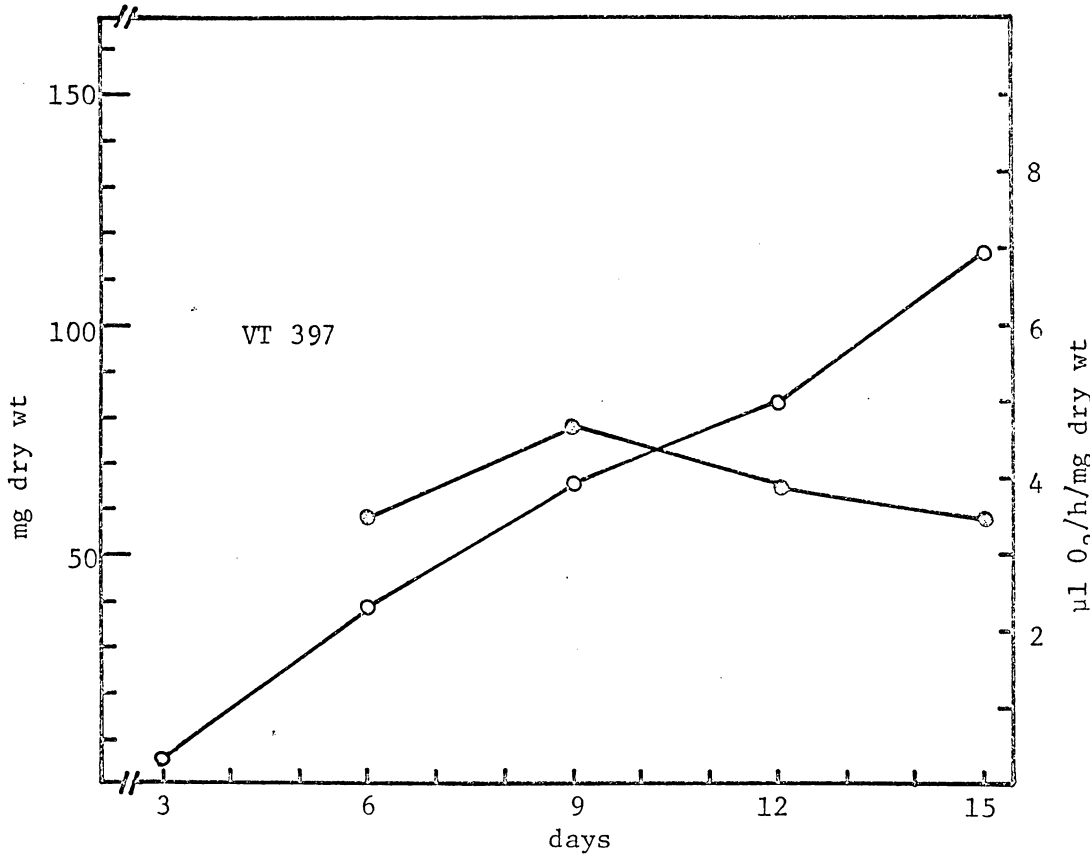

Figure 3. Combined dry weight and oxygen uptake data from Pholiota limonella (VT 397)

0 = oxygen uptake
0 = dry weight

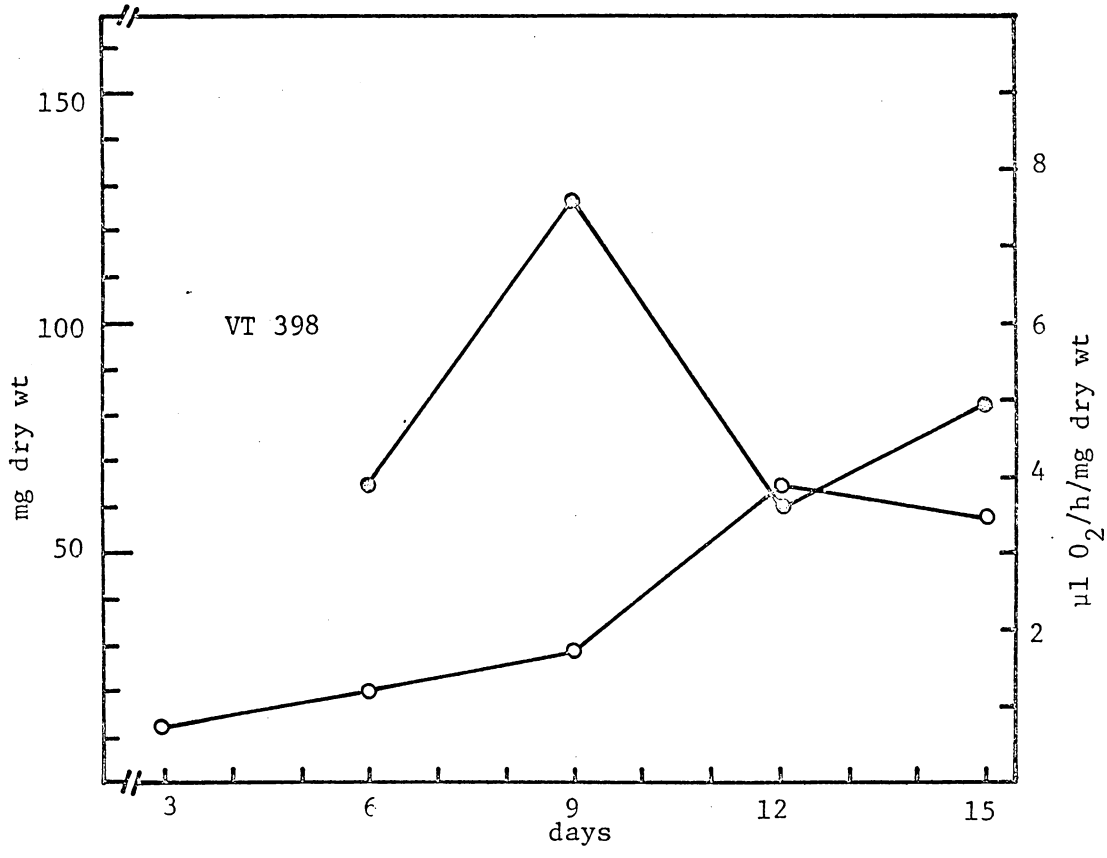

Figure 4. Combined dry weight and oxygen uptake data from Pholiota aurivella (VT 398)

⊗ = oxygen uptake
0 = dry weight

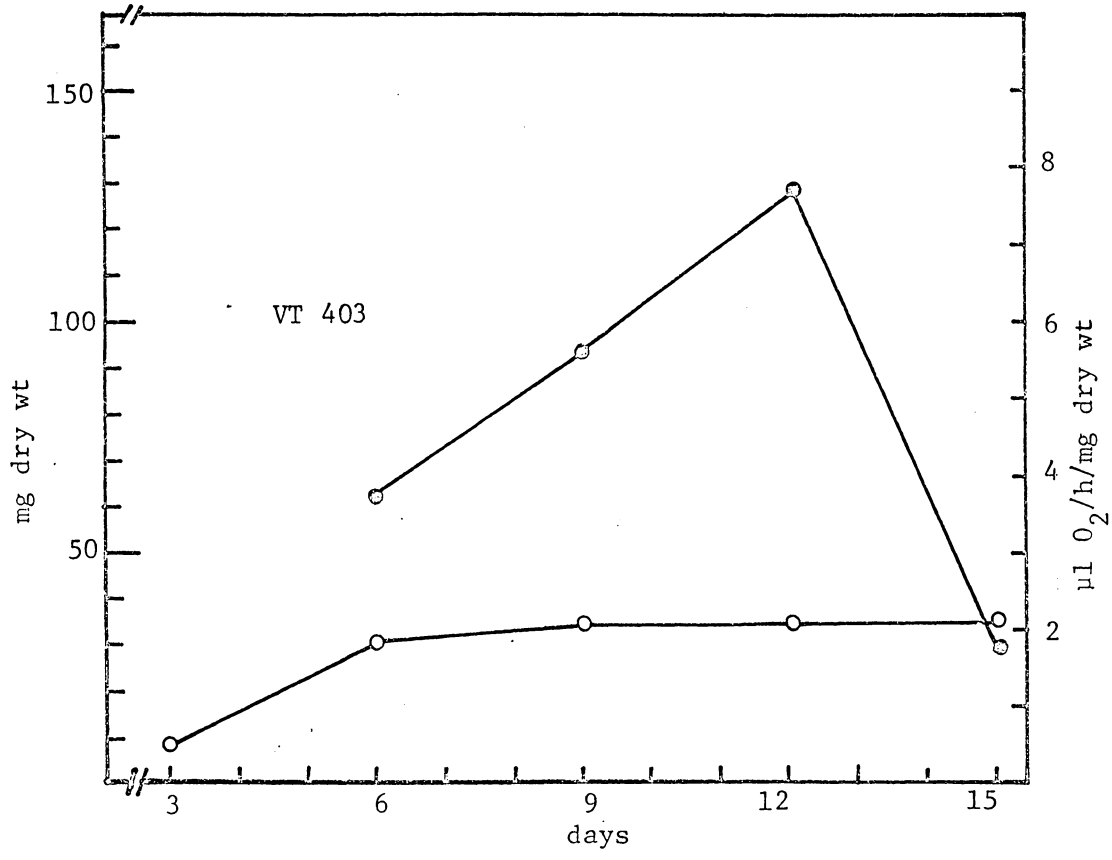

Figure 5. Combined dry weight and respiration oxygen uptake data from Pholiota limonella (VT 403)

● = oxygen uptake
0 = dry weight

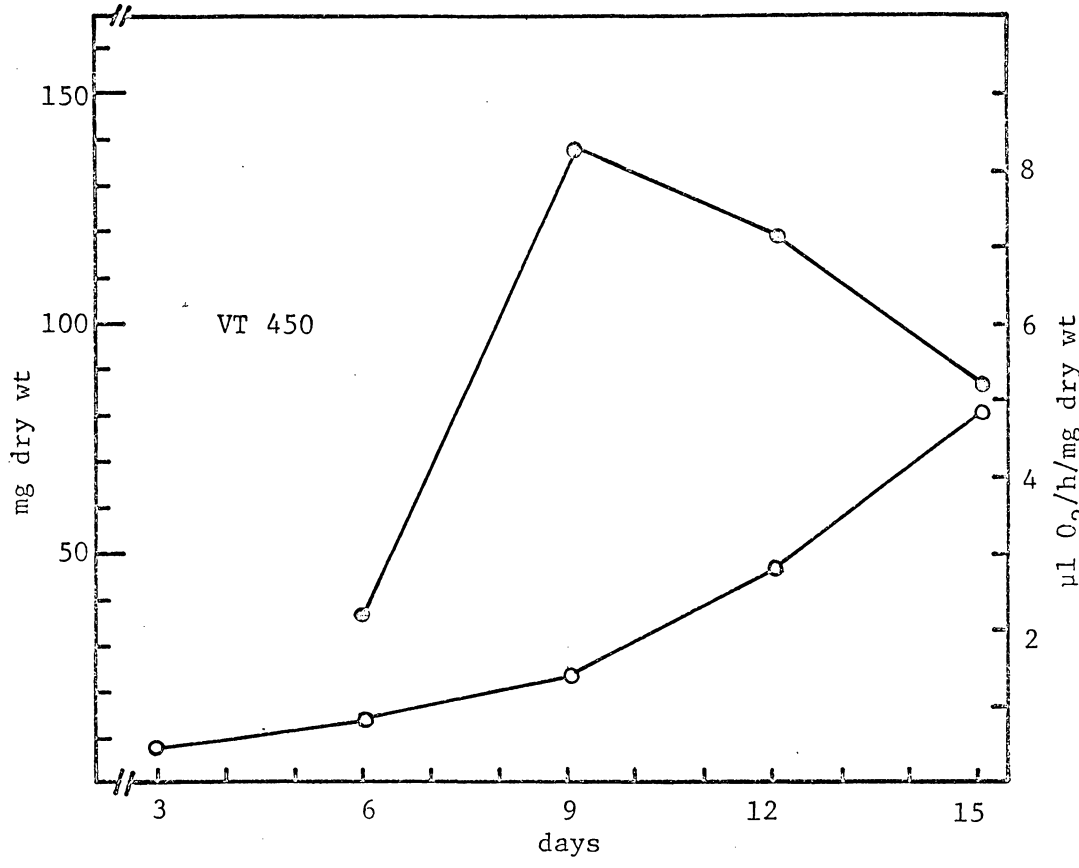

Figure 6. Combined dry weight and oxygen uptake data from Pholiota limonella (VT 450)

● = oxygen uptake
O = dry weight

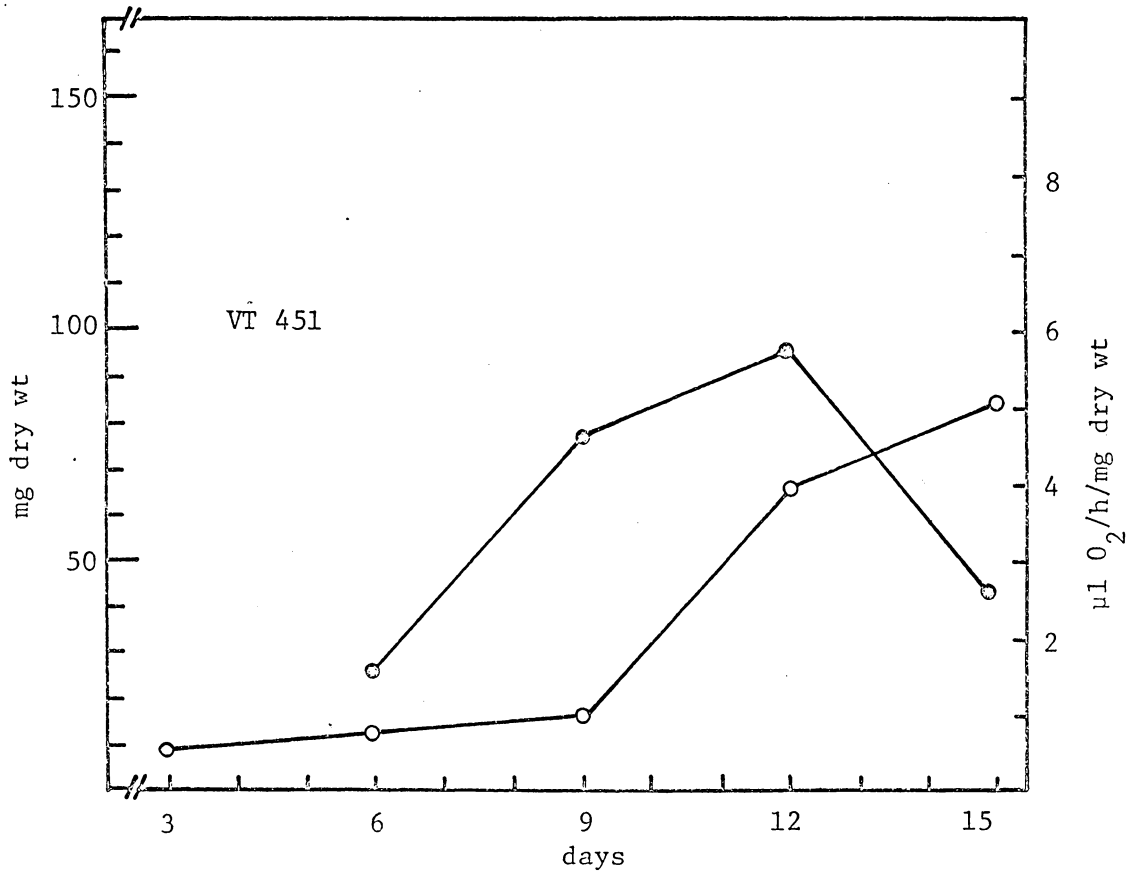

Figure 7. Combined dry weight and oxygen uptake data from Pholiota limonella (VT 451)

● = oxygen uptake
0 = dry weight

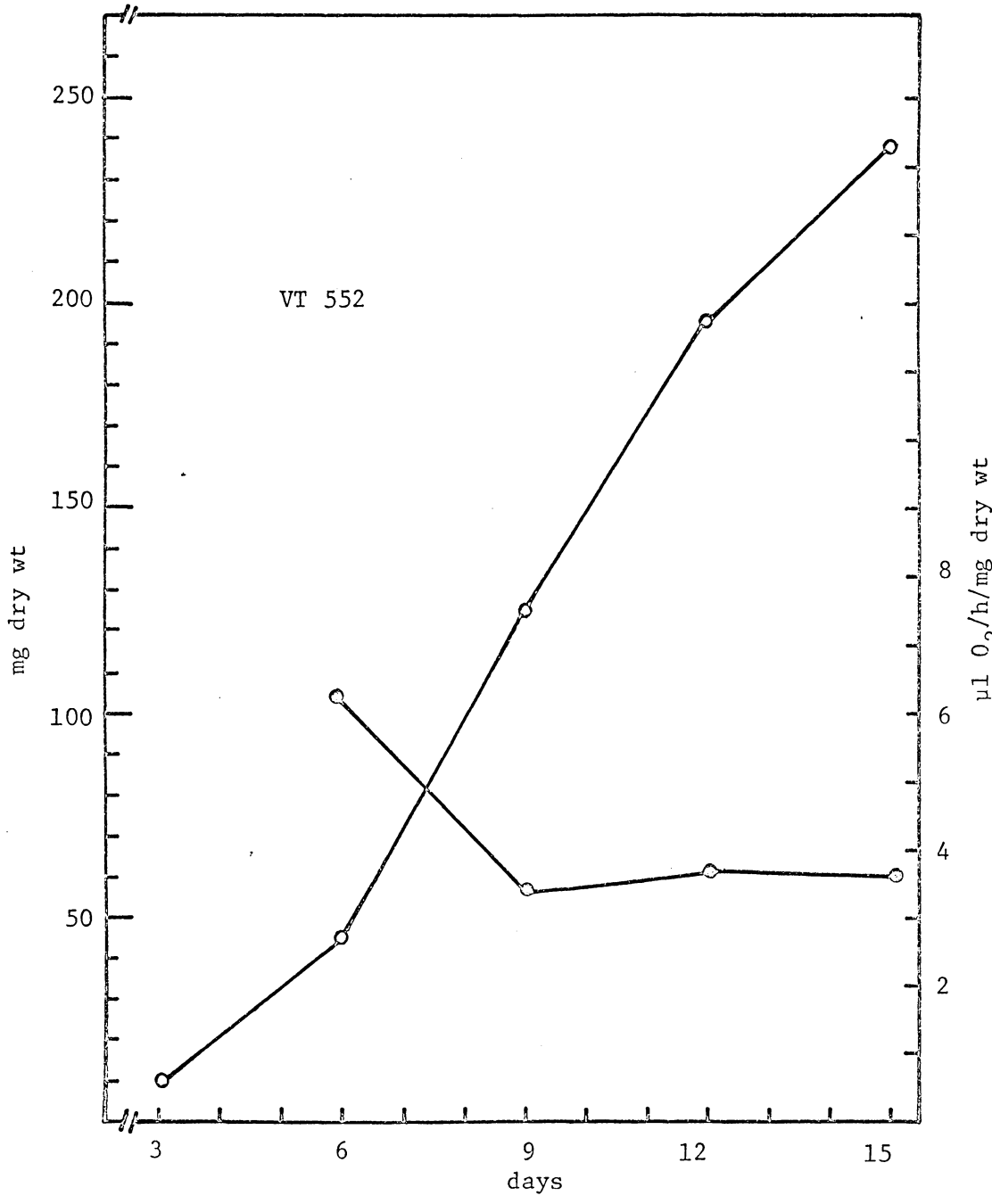

Figure 8. Combined dry weight and oxygen uptake data from Pholiota aurivella (VT 552)

● = oxygen uptake
○ = dry weight

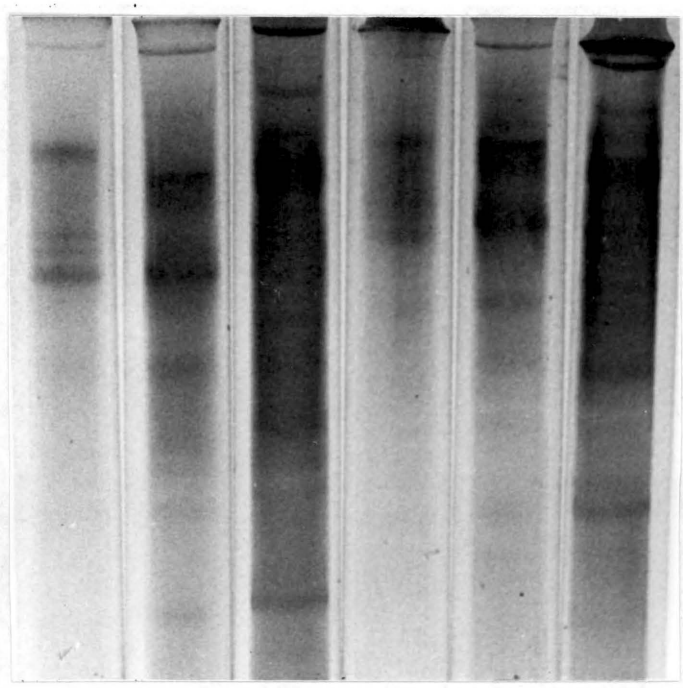

Figure 9. Comparisons between different gel runs and different growth batches of general protein patterns.

a = grown in September
b = grown in October
c = grown in November
d, e, and f are reruns of a, b, and c.
All samples are Pholiota limonella, VT 395.

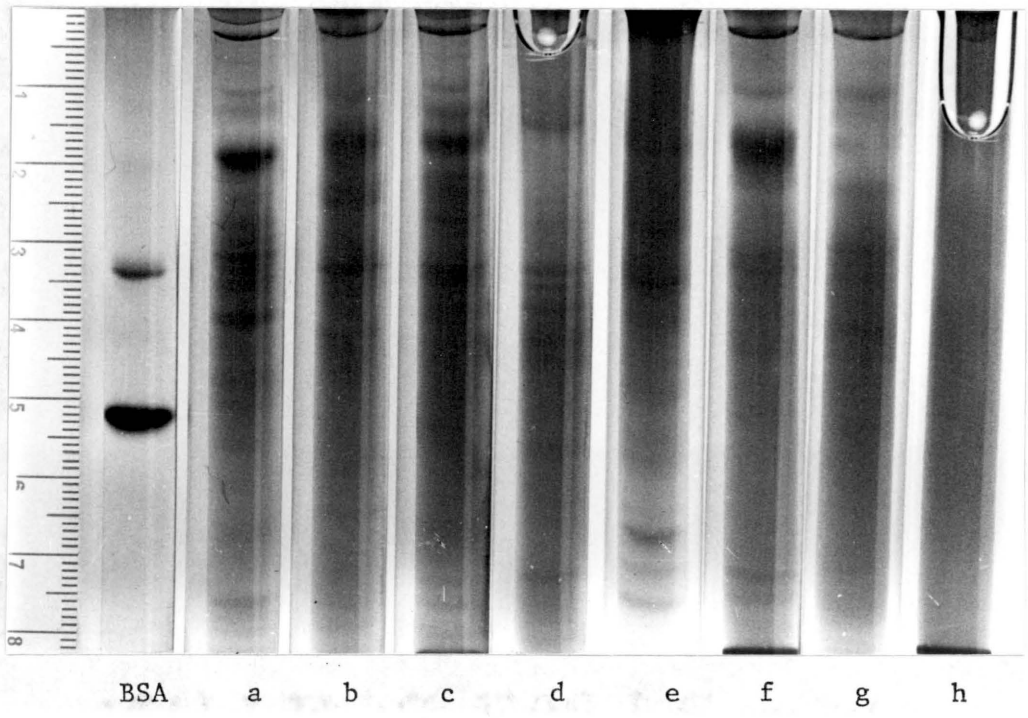

Figure 10. General protein patterns of all isolates.

BSA = bovine serum albumine
a = VT 394
b = VT 395
c = VT 397
d = VT 398
e = VT 403
f = VT 450
g = VT 451
h = VT 552

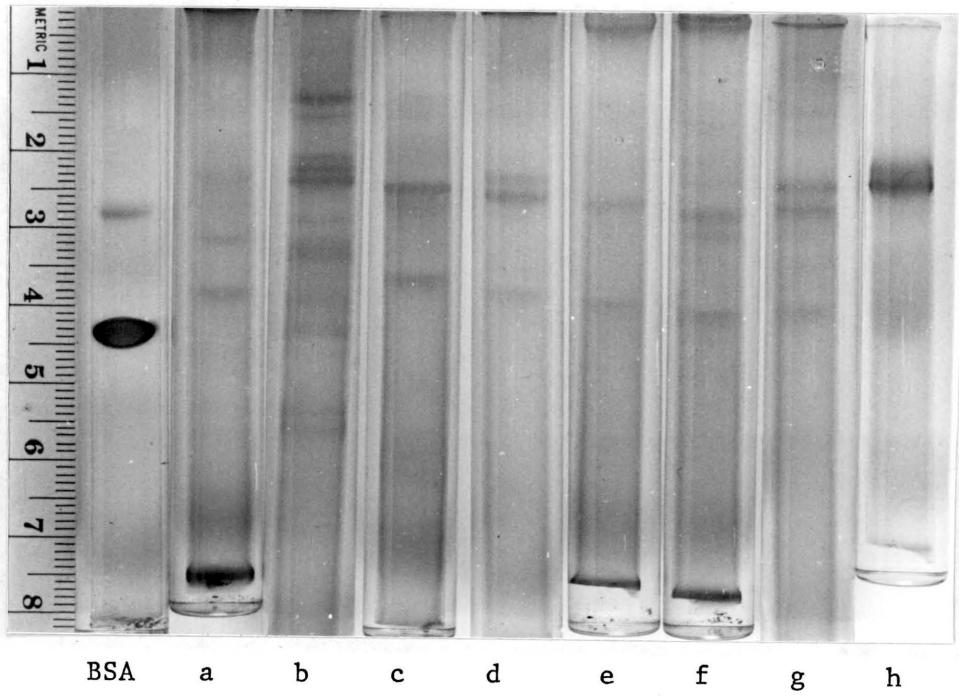

Figure 11. General proteins patterns of all isolates, after butanol treatment.

BSA = bovine serum albumine
a = VT 394
b = VT 395
c = VT 397
d = VT 398
e = VT 403
f = VT 450
g = VT 451
h = VT 552

30 species of higher fungi, among them Pholiota mutabilis and Pleurotus ostreatus, and proved the hypothesis that phenoloxidases take part in the early degradation of lignin, white rot fungi forming large amounts of extracellular phenoloxidases.

Nobles (1958) proposed that the capability of a species to produce extracellular oxidases was of taxonomic significance and divided Polyporaceae in those species that caused brown rot and produced no extracellular oxidases and to white rot species that produced extracellular enzymes. The tests were carried out on cultures grown on malt agar supplemented with tannic or gallic acid and the discolored zones on a background of light colored agar were visually easy to record. The oxidase producing species were able to degrade lignin.

Madhosingh (1970) examined tyrosinases by electrophoresis in several agarics, e.g. in Pholiota aurivella. He found seven major DOPA-catalytic components as recorded in densitometric readings, but warned about the use of enzyme patterns in species characterization because of the variation observed in the experiments. The electrophoretic technique was found valuable in separating different tyrosinases, but not valuable in comparing heterogenous isolates.

In this study all isolates showed tyrosinase activity, the main band being from 26.2 to 31.0 on a scale where the marker dye is considered 100 (=Rf). There were no prominent differences between the isolates. In addition to the main bands there were observed some minor traces of activity on four isolates: a, b, c, and e (VT 394, VT 395, VT 397, and VT 403), Figure 12, page 40. These minor bands

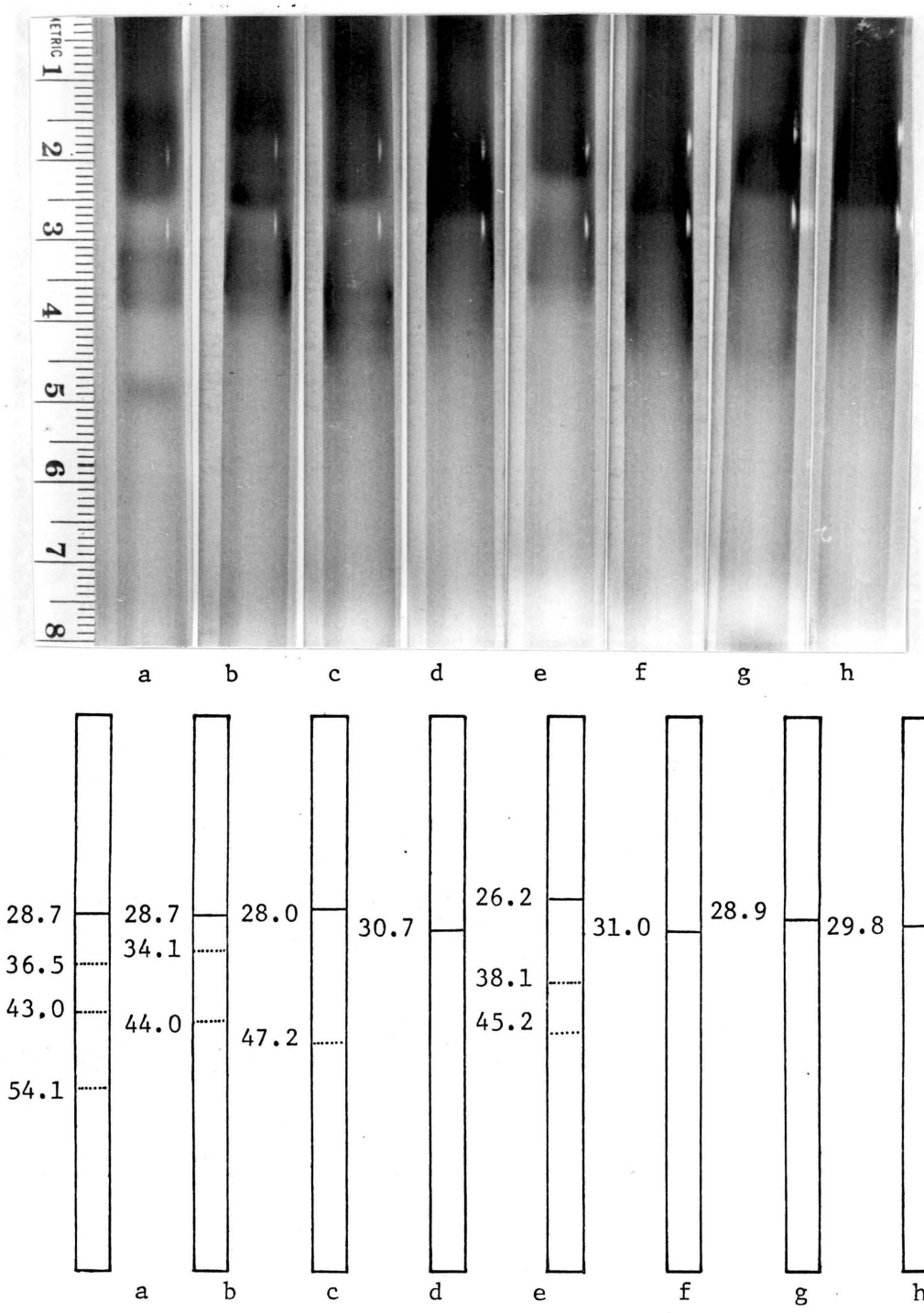

Figure 12. Tyrosinase zymograms

a = VT 394
b = VT 395
c = VT 397
d = VT 398
e = VT 403
f = VT 450
g = VT 451
h = VT 552

Numbers beside the drawings represent the relative migration of enzymes, when the marker dye is 100.

were not stable, and could not be repeated in a rerun after storage. Both Pholiota aurivella isolates, VT 398, and VT 552 (d and h, Figure 12) did not show these minor bands, but also two of the P. limonella strains VT 450 and VT 451, (f and g) could be included in this category.

All isolates showed activity for laccase substrate and the activity was shown as either wide areas as in d, e, g, and h (VT 398, VT 403, VT 451, and VT 552), Figure 13, page 42, including both P. aurivella strains, or as two wide, but distinctly separate bands as in a, b, c, and f (VT 394, VT 395, VT 397, VT 450), all P. limonella strains. These wide areas of activity did not show patterns that could have been used in the species delineation.

3.5 Esterases

Esterases are defined in this work as those compounds that show activity when alpha-naphthylacetate is used as substrate (carboxylic ester hydrolases E.C. 3.1.1; Peberdy and Turner, 1968). Esterases are among the most common group of enzymes that have been used as aid in taxonomy. Meyer et al. (1964) studied Fusarium oxysporum (Schl.) emend. Snyder and Hansen and F. xylarioides Steyaert and their forma speciales and was able to characterize different taxa in this way. Schánĕl et al. (1971) showed distinct esterase patterns in each Trametes strain studied. Reddy and Threlkeld (1971) found esterase patterns helpful in characterizing six out of eight studied Neurospora species. The esterases from Mortierella ramanniana (Moeller) Linneman (Perberdy and Turner, 1968) displayed very variable patterns and did not prove helpful in taxonomy. Also Moorhouse and

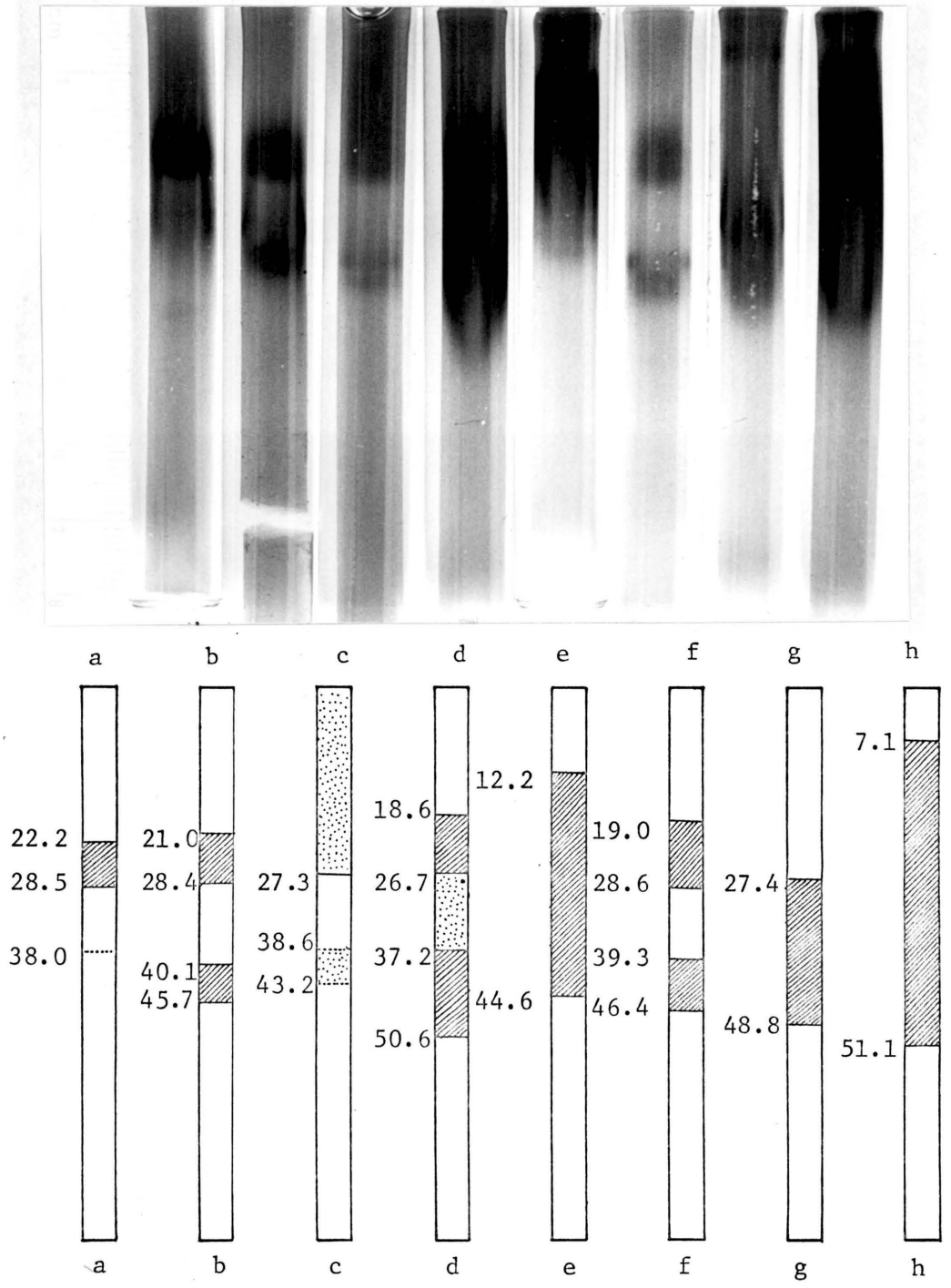

Figure 13. Laccase zymograms

a = VT 394
b = VT 395
c = VT 397
d = VT 398
e = VT 403
f = VT 450
g = VT 451
h = VT 552

Numbers beside the drawings represent the migration of enzymes, when the marker dye is 100.

de Bertoldi (1975) found a wide spectrum of esterase patterns in a study of genus Humicola Traaen. Blaich (1977) found esterases most suitable for characterizing species in a survey of enzymes as aid in taxonomy for higher Basidiomycetes, e.g. Pholiota aurivella showed seven bands of exterase activity, and he compared esterase bands to fingerprints. Lloyd et al. (1972) found esterase only at different stages of conidiation in Aspergillus and suggested that esterases are found only at the declining stages of growth in the utilization of lipids as carbon source. In this work conidia were observed at all isolates and they were already present after three days of growth in the starter flasks, so it cannot be suggested that the presence of conidia would indicate growth past logarithmic phase. Esterase may vary, though, as indicated studies by Wang and Raper (1970) when they found six major bands and two weak ones in a wild monokaryon of Schizophyllum commune, but the major bands were reduced to three with three minor bands accompanying them after dikaryozation of the same monokaryon. Esterases were present at all stages of sclerotium formation in Sclerotium rolfsii, but there was only one fraction that was active from initiation till the formation of sclerotia. The mycelium displayed four distinctive active areas (Chet et al., 1972). When Schanel et al. (1971) harvested Trametes species after three and ten days of growth, the intensity of the bands increased with the incubation time, but the overall activity was the same.

All isolates in this study showed activity for alphanaphtyl acetate. There were two separate areas of activity: from (Rf) 7.9 to 17.8 and from 43.8 to 57.5. The upper area was weak or absent in a, e, f, and h (VT 394, VT 403, VT 451, and VT 552) in Figure 14,

page 45, whereas b, c, d, and f (VT 395, VT 397, VT 398, and VT 450) displayed two strong areas of activity. It can be seen here that Pholiota aurivella (VT 398, and VT 552) could not be separated from P. limonella isolates in this way.

3.6 Peroxidases

Peroxidases are common metabolic enzymes that take part in various reactions in organisms by breaking down hydrogenperoxide. They have often been used in the characterization of various organisms: Clare et al. (1968); Reddy and Threlkeld (1971); Schanel et al. (1971); Chet et al. (1972); Shannon et al. (1973); Moorhouse and de Bertoldi (1975), but peroxidase have not been as good as e.g. esterases in defining taxa. The relative easy performance of the tests might have contributed to the common use of it and the results of these tests have been reported when results of other enzyme tests have been published. Reddy and Threlkeld (1971) found no variability in peroxidases in their study of eight species of Neurospora: all isolates showed three sites of activity, when pyrogallol and H_2O_2 were used as reagents. Schánĕl et al. (1971) reported stability of peroxidases when samples were taken from cultures of different age of Trametes versicolor and T. hirsuta. When H_2O_2 and benzidine were used as reagents peroxidases were found only in mature sclerotia of Sclerotium rolfsii (Chet et al., 1972).

In this work catechol and pyrogallol were used with hydrogen peroxide to test the peroxidase and catechol oxidase activity. Both test acted similarly, showing only one area of activity that was difficult to record accurately because of the developing stain diffused

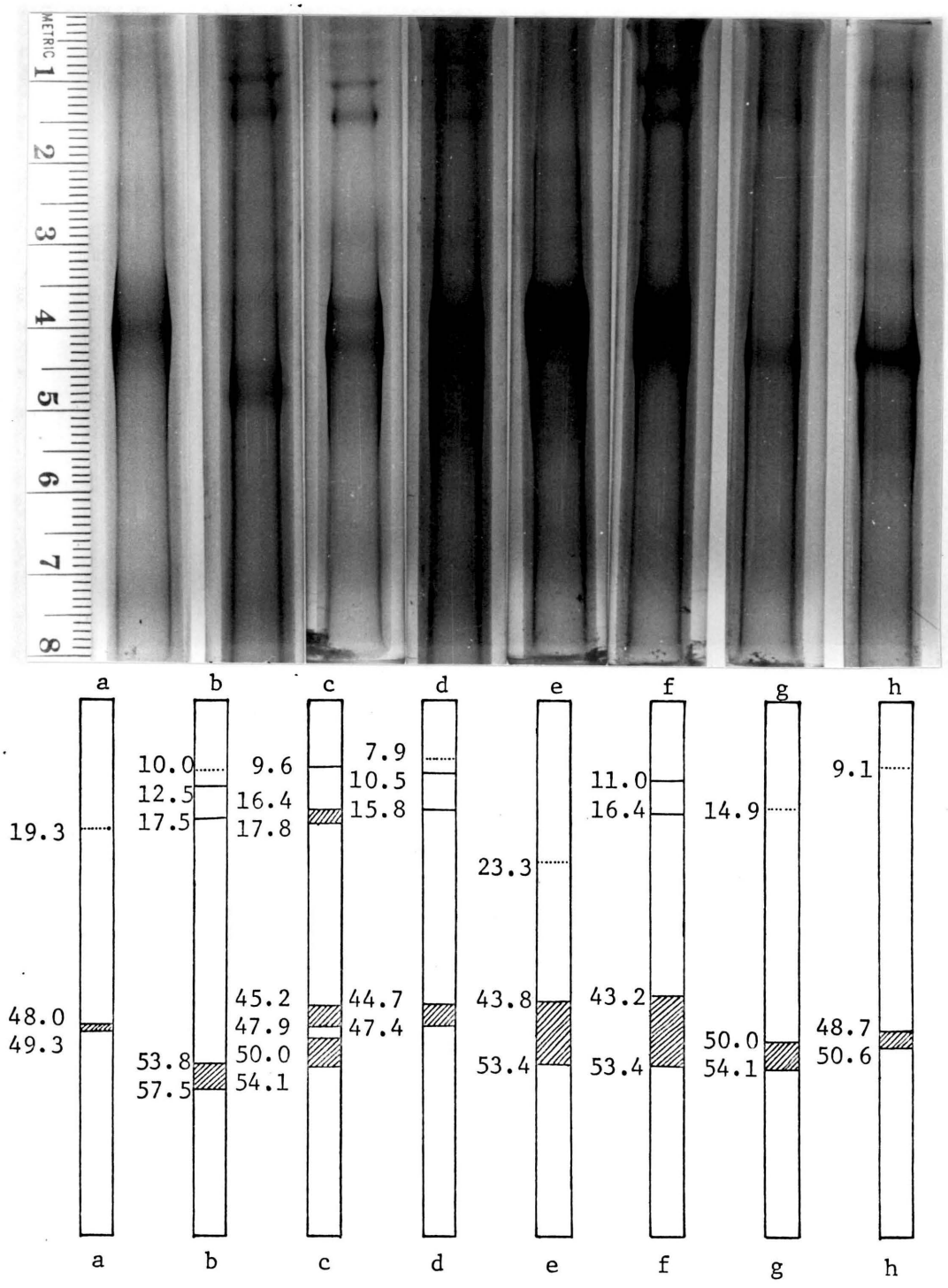

Figure 14. Esterase zymograms

a = VT 394
b = VT 395
c = VT 397
d = VT 398
e = VT 403
f = VT 450
g = VT 451
h = VT 552

Numbers beside the drawings represent the relative migration of enzymes, when the marker dye is 100.

to the gel very rapidly. Results of catechol oxidase tests are reported in drawings in Figure 15, page 47. The center of the band in each isolate fell between 18.4 and 23.7 (Rf) and the only difference between isolates was the intensity of the reaction. Pholiota limonella strains, b, e, and f (VT 395, VT 403, and VT 450) reacted most strongly to catechol, whereas a, c, and h (VT 394, VT 397, and VT 552) gave some reaction, and d, P. aurivella (VT 398) and g, P. limonella (VT 451), showed almost none. The activity of the enzymes to pyrogallol was more even so that only VT 451 gave a very weak reaction, all others were comparable to the commercial standard used (Figure 15, page 47).

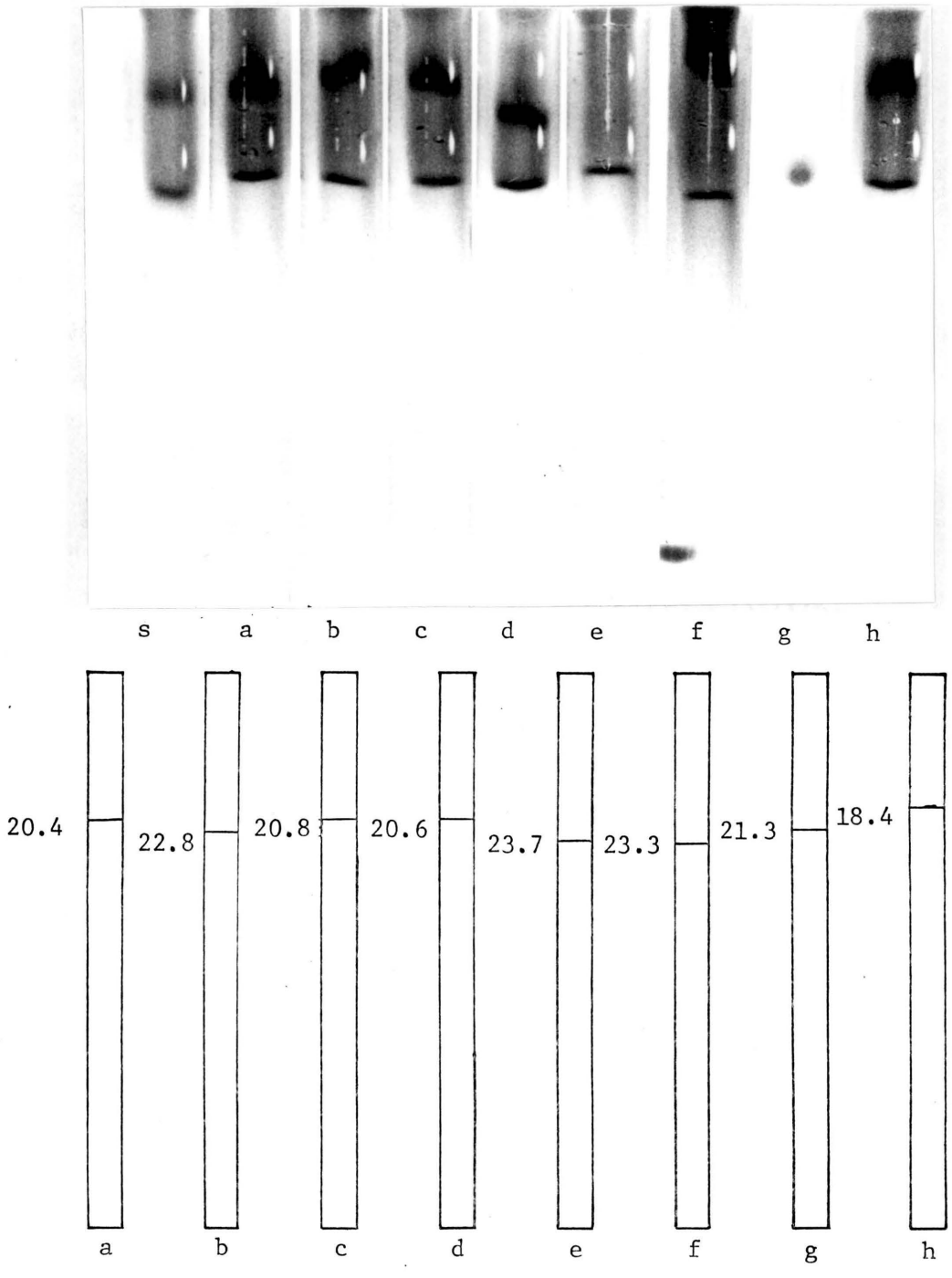

Figure 15. Peroxidase zymograms

a = VT 394
b = VT 395
c = VT 397
d = VT 398
e = VT 403
f = VT 450
g = VT 451
h = VT 552
s = standard

Numbers beside the drawings represent the migration of enzymes, when the marker dye is 100.

4. Discussion

The purpose of this study was to seek chemotaxonomic evidence to separate two closely related species, Pholiota limonella and P. aurivella, that earlier (Farr et al., 1977) were shown to be distinguishable by the mating studies. Genes express themselves by coding for characteristic proteins. By describing those proteins one draws a picture of the genetic material that produced them. One type of evidence, genetic, should then support the other evidence, chemotaxonomic (Sibley, 1962). Since enzyme patterns have been shown to be valuable in fungal taxonomy (e.g. Clare et al., 1968; Franke, 1973; Snider, 1973), this chemotaxonomic approach was used for evaluation of Pholiota species. Especially esterases and phenoloxidases have been documented to separate species in higher fungi (Nobles, 1958; Molitoris, 1977; Blaich, 1977).

Polyacrylamide gel electrophoresis of the proteins in the studied Pholiota isolates did not show banding patterns that could have separated the isolates of one species from those of the other. Repeated runs of extracts from different growth batches gave characteristic patterns in regard to positions and numbers of bands, but they did not aid in differentiating taxa.

Tyrosinases showed one wide area of main activity and there were not found any differences between isolates. Laccase activity showed up in two distinct areas and there were differences in the intensity of the reactions between isolates, but it could not be

correlated to the two species studied here. Esterase patterns displayed distinct bands in two main areas and each of these areas had one to three active bands. These banding patterns gave no consistent differences in numbers or positions that could support the separation of these isolates in different species. Peroxidases showed only one area of activity and no conclusions could be made about their positions in the gels.

It is apparent then that general protein and isozyme patterns used here do not support separating strands of P. aurivella and P. limonella at species level. It may be possible that there are other enzyme systems that could be more specific to a given group of isolates or that SDS gel electrophoresis could give more information for the specific placement of these polymorphic fungi. The method itself should be studied very carefully for each material under investigation so that possible sources of variation could be diminished.

5. Conclusions

1. Polyacrylamide gel electrophoresis of soluble proteins in Pholiota aurivella and P. limonella did not display distinctive banding patterns that could have been interpreted as species specific.
2. When electrophoresed protein extractions were stained for esterase activity using alpha-naphthylacetate as substrate, no clear differences could be found between isolates of P. aurivella and P. limonella.
3. Phenoloxidases, laccases and tyrosinases, showed activity in all studied strains, but there were no consistant differences between isolates.
4. Peroxidases showed wide areas of activity and it was difficult to determine the exact bands for these enzymes.
5. None of the chemotaxonomic approaches used here to separate two polymorphic species could further delineate the taxa.

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ISOZYME PATTERNS OF SELECTED ISOLATES OF *PHOLIOTA* IN THE STIRPS "*ADIPOSA*"

by

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(ABSTRACT)

The species concept in *Pholiota*, stirp *Adiposa* Smith and Hesler, has been investigated by applying electrophoretic techniques to isolates that have previously been examined for their morphology and mutual mating compatibility. Crude protein extracts of soluble proteins from mycelium grown in liquid shake culture were used to obtain general protein patterns and zymograms of esterases, (alpha-naphthylamidases), phenoloxidases (laccases and tyrosinases), and peroxidases on polyacrylamide gels. Harvesting of different isolates for protein extraction was carried out at comparable stages of development using dry weight determinations and respiration studies as criteria. Peroxidase and phenoloxidase zymograms showed wide areas of activity and no distinct banding patterns were attained. Esterase zymograms displayed characteristics that made it possible to arrange the studied isolates in separate groups, but this classification did not completely follow results from the previous work. The chemotaxonomic approach to the species complex of *P. aurivella* (Fr.) and *P. limonella* (Pk.) Sacc. showed that the species are closely related and cannot clearly be separated in this way.