Enzyme activity of mycelial cultures of saprotrophic macromycetes (Basidiomycotina). III A taxonomic application

Enzymatická aktivita myceliových kultur saprotrofnich makromycetů (Basidiomycotina). III Využití v taxonomii

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Mycelial cultures of 92 species belonging to 40 genera of saprotrophic basidiomycetous fungi (orders *Agaricales, Aphyllophorales, Gastrosporiales, Lycoperdales* and *Nidulariales*) were tested with respect to the production of extracellular hydrolytic enzymes and oxidoreductases (laccase, peroxidase, tyrosinase, diaminooxidase, proteases, amylases, urease, p-cresol oxidases and hydrolyse of tyrosine) using simple plate and spot tests. The results obtained were evaluated by means of factor analysis methods. Distribution of enzyme activities in individual species was discussed.

Myceliové kultury 92 druhů saprotrofních basidiomycetů (z řádů *Agaricales, Aphyllophorales, Gastrosporiales, Lycoperdales* a *Nidulariales*) byly otestovány jednoduchými plotnovými a kapkovými testy na produkci extracelulárních hydrolytických enzymů a oxidoreduktáz (lakkázy, peroxidázy, tyrosinázy, diaminooxidázy, proteáz, amyláz, ureázy, p-kresol oxidáz a hydrolýzy tyrosinu). Výsledky jsou zpracovány pomocí metod faktorové analýzy. Diskuse je zaměřena na distribuci enzymatických aktivit u jednotlivých druhů.

Introduction

The present work is an extension of the previous studies (Klán and Baudišová 1990a, 1990b) in which the enzyme activity of mycelial cultures of saprotrophic macromycetes was investigated. In the first two parts the methods of plate diffusion and spot tests were elaborated including the study of literature data and evaluation of possible uses of individual detection agents. In the present work ten enzyme activities (oxidoreductases and hydrolases) were tested in mycelial cultures of 92 species (43 genera) of *Basidiomycotina* belonging to five orders (*Agaricales, Aphyllophorales, Gastrosporiales, Lycoperdales, Nidulariales*). In forty species the enzyme activities have not yet been investigated. The present study thus represents the most extensive study screening with respect to the heterogenity of species and enzymes studied. The results could serve for more detailed chemotaxonomic studies of individual taxons or for selection of species for a further biochemical study.

In an extensive study of oxidoreductases in mycelial cultures of wood-decaying fungi Käärik (1965) tested a collection of *Basidiomycetes*. Das et al. (1979) tested the production of certain hydrolases (e.g. amylases or proteinases) in 25 polyporous species and 19 enzyme activities (esterases, aminopeptidases, oxidases) were studied by Fiasson and Bernillon (1983) in mycelial cultures of 36 polyporous species. A larger collection of *Basidiomycetes* was used in their studies of proteolytic enzymes e.g. by Buchalo et al. (1971) - 36 species or by Mišurcová et al. (1987) - 91 species.

Material and Methods

All cultures were from the Culture Collection of Fungi of the Institute of Toxicology, Faculty of Medicine, Charles University in Prague (Klán and Štípek 1987). Before testing the fungi were precultivated on Petri dishes with malt extract agar (Imuna) containing malt extract 35 g, bactopeptone 5 g, agar 13 g in 1 l of double distilled water, pH 6 - 6.5.

Enzyme tests were described in detail previously (Klán and Baudišová 1990a - hydrolases, Klán and Baudišová 1990b - oxidoreductases).

Studied enzymes:
Oxidoreductases: 1. Spot tests: laccase (syringaldazine as substrate), peroxidases (p-phenylenediamine tartrate and 3% hydrogen peroxide), diaminooxidase (ethyloxethyl-p-phenylenediamine, photographic developer Agfa T 32), "phenoloxidase" = p-cresol oxidases (p-cresol):
Plate diffusion methods: tyrosinase (L-tyrosine).
Hydrolases: 1. Plate diffusion methods: amylase (starch and Lugol solution), proteases (casein or gelatine), hydrolyse of tyrosine (L-tyrosine), urease (urea and phenol red.).
In most species 2-3 strains were tested. However, as the results did not quantitatively differ, only a single species is presented here (as OTU). Differences in the quantity of individual enzymes produced by several strains of a single species cannot be excluded. However they could not be detected accurately, due to the semiquantitative nature of the tests. Some statistic methods of the program block Statgraphics, version 2.6, were used for the treatment of the results. Factor analysis with rotation method Varimax, convergence criterion 10^{-3}, was used as a decreased dimension method, properties were not standardized. "Sun ray plots" method was only used as a trial procedure for a simple expression of similarities of enzyme activities in selected species.

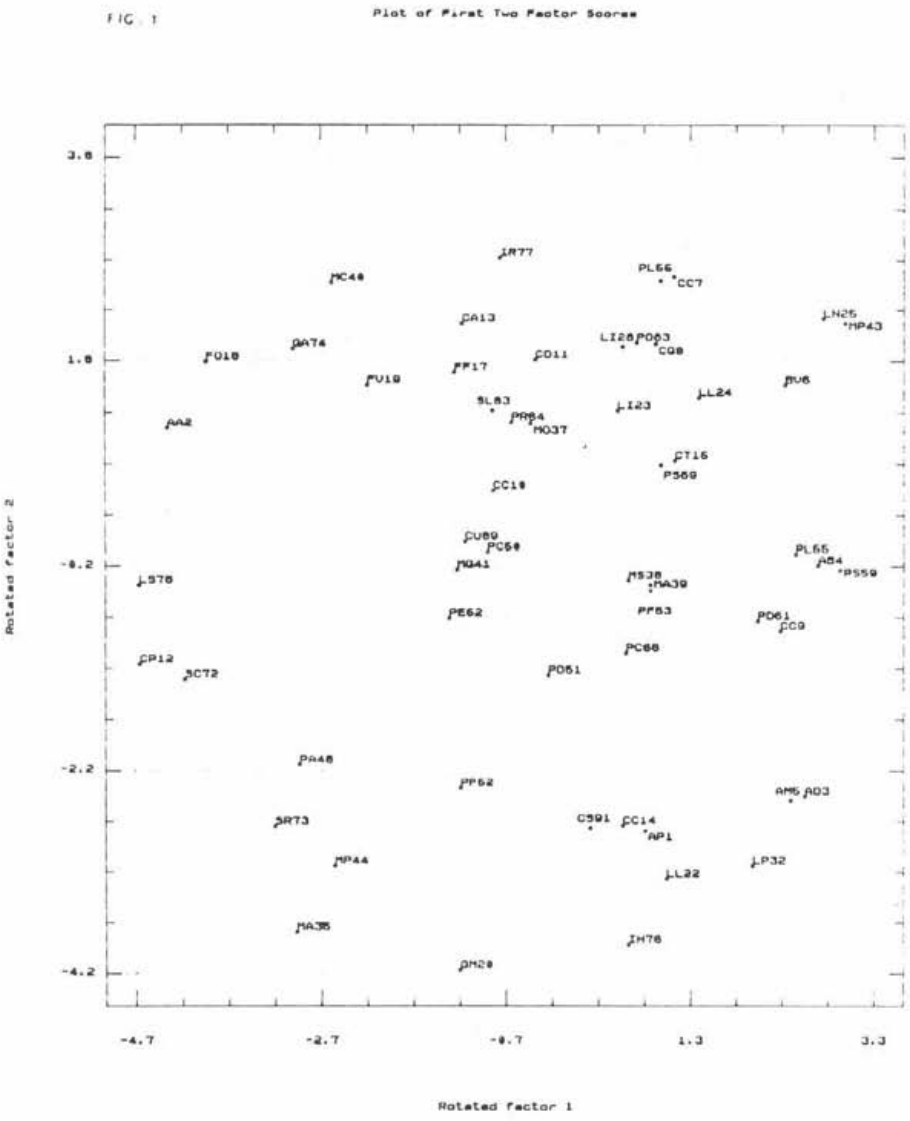
Results and Discussion

The results concernig enzyme acitivities in 92 species of *Basidiomycotina* are summarized in Table 1. The species are arranged alphabetically according to the system *Agaricales, Aphyllophorales, Gasteromycetes*. For a simple evaluation of the occurrence of enzymatic activities in individual fungal species the method of factor analysis was used. Due to the number of the fungal species studied and to binary coded properties a number of objects of graphs of the first two factor after rotation overlap (see Fig. 1). In the Fig. 2 the studied properties (enzymes) are projected again into the plane of the first two factors after rotation. The distance of the points in the plane indicates their mutual similarity. Plots of the first two factor scores (for species) and weights (for properties) saturate 45.3% of the overall variability.

Sun ray plots (see Fig. 3) show a graphically demonstrated occurrence of enzyme activities. The method was only used for an optical comparison of a mutual similarity of species on the basis of their enzyme apparatus in selected examples (family *Tricholomataceae)*.

It follows from the results obtained in 92 species of *Basidiomycotina* that the studied enzyme activities occur rather frequently in mycelial cultures. If 10 enzymes were studied that would be completely independent, 1024 combinations (2^{10}) should be observed which should be sufficient for determination of 9^2 species. However, the activities are interconnected and, hence, in a number of cases objects in the Figure of the first two

FIG. 1


Plot of First Two Factor Scores
Rotated factor 2
Rotated factor 1
3.8
1.8
-0.2
-2.2
-4.2
-4.7
-2.7
-0.7
1.3
3.3
AR77
PC48
PL66
CC7
PA13
LN25
MP43
LI28
PO63
CG8
PA74
PO18
CO11
FF17
PV19
PV6
SL83
LL24
LI23
PR84
MO37
AA2
PT16
P569
PC19
PU89
PC69
PL65
AB4
MQ41
MS38
MA39
PS59
LS76
PE62
PF83
PO61
CC9
PC66
CP12
AC72
PO51
PA48
PF62
AM5
AO3
AR73
C591
CC14
AP1
MP44
LL22
LP32
MA36
IH76
PH28

factors overlap after rotation (Fig. 1). A total of 56 combinations of enzyme activities occurred, of them 35 points represented a single species (e. g. all three *Pleu-rotus* species investigated). The presence of all studied enzyme activities and the absence of the hydrolytic degradation of tyrosine was the most frequent combination being observed in 7 species (*Bolbitius vitellinus* BV6. *Lepista nuda* LN26. *L. personata* LS27. *L. sordida* LS29. *Oudemansiella mucida* OM 45. *Pholiota spumosa* PS58. *Polyporus rhizophilus* PR81). Five species (e. g. *Calocybe gambosa* CG8, *Pholiota adiposa* PA47, *P. cerifera* PA49, *P. gummosa* PG54, *P. jahnii* PM57) did not produce peroxidase, urease and did not hydrolyse tyrosine. One tetrad, five trinities and 13 species pairs with an identical enzyme combination also occurred.

If we realize that the distance of the points on the plane reflects their mutual similarity it cannot be concluded that a certain enzyme apparatus would be characteristic for individual taxons or ecological groups (e.g. polyporous *Ischnoderma resinosum* has the same enzyme apparatus as does the steppe hypogeic gasteromycete *Gastrosporium simplex*). The only taxon which is relatively homogenous with respect to enzyme activity appears to be the genus *Lepista* (7 species). In the collection studied activities of proteolytic enzymes (ENZGEL, ENZCAS) are significantly positively associated. They also represent a relatively isolated group with respect to the enzyme activities investigated. Therefore, the proteolytic activity is a significant chemotaxonomic marker and should be determined in addition to oxidase tests that have so far been performed much more frequently. However, it is sufficient to use only one of the substrates (Klán and Baudišová 1990a). Terminal oxidases, i.g. laccase (syringaldazine as substrate), tyrosinase (L-tyrosine) and peroxidase (p-phenylendiaminetartrate and hydrogen peroxide) are independent on each other. P-cresol used sometimes for determination of non-specific tyrosinase (Käärik 1965, Marr 1979) but also oxidized by laccase is positively associated with laccase and tyrosinase (Fig. 2). The factor analysis indicates that oxidation and hydrolysis of tyrosine are mutually independent processes as the points of these activities (ENZTYR and HYTYR) are quite distant in Fig. 2. Catalase, glucose-2-oxidase and lipase (tributyrin) are not presented here as the results of tests were all positive. In addition, the results of milk clotting enzymes tests are also not presented here as in this case the results obtained in different strains of the same species were sometimes different.

The spectrum of the enzymes presented here (laccase, tyrosinase, peroxidase, amylase, proteases, urease, diaminooxidase, hydrolyse of tyrosine) occasionally supplemented by some other enzyme activities involved in decomposition of natural substrates (cellulases, pectinases, xylanases, ligninases) should be sufficient for characterization of a larger, heterogenous collection of mycelial cultures of fungi. A detailed study should include a

FIG 2 Plot of First Two Factor Weights

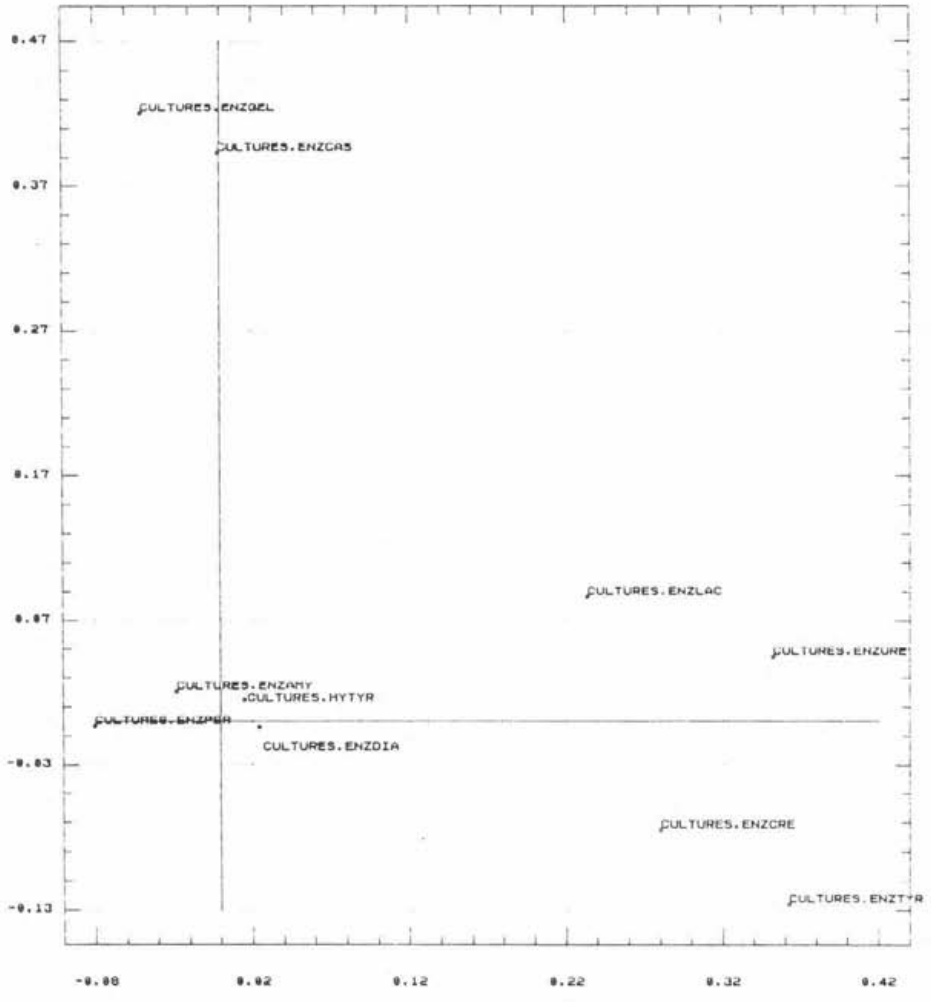
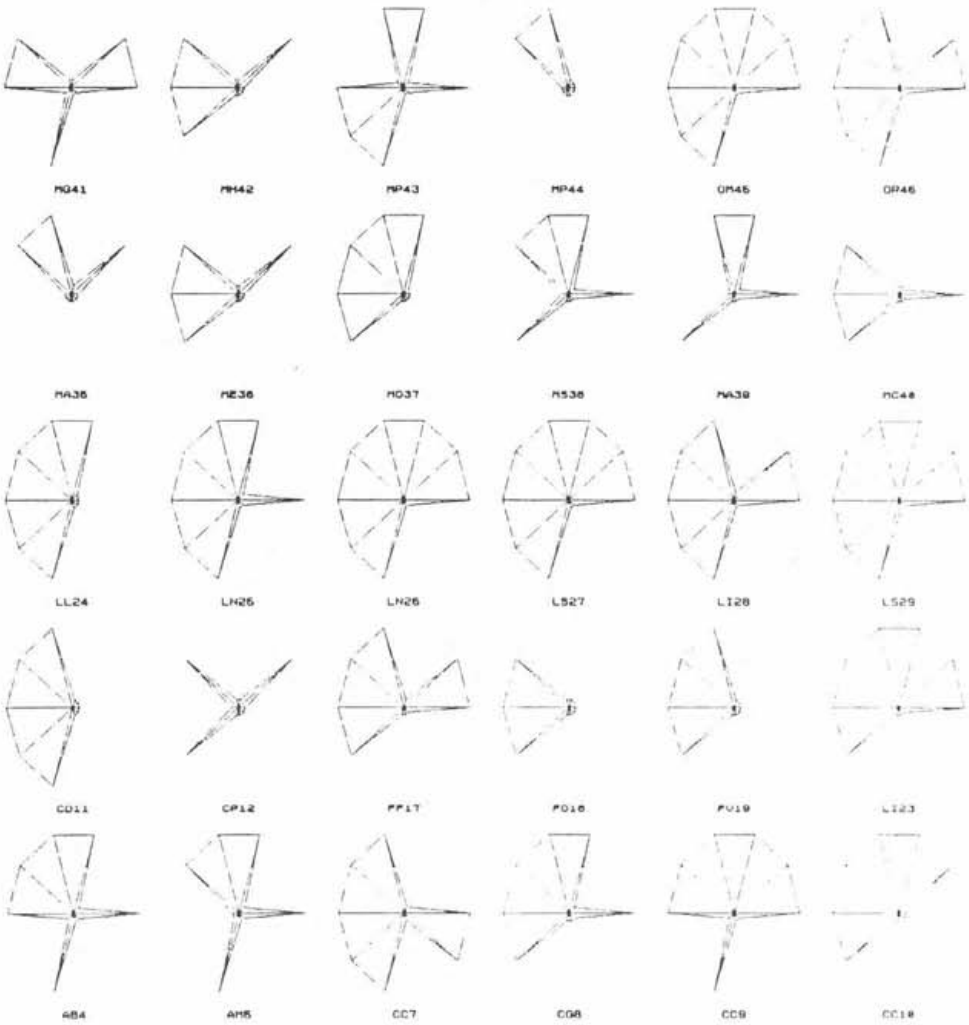
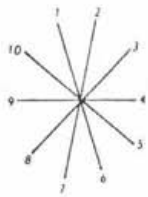


FIG. 3

STAR SYMBOL PLOTS

Family Tricholomataceae





Explanatory notes: 1.- p-cresol oxidases, 2.- tyrosinase, 3.- peroxidase, 4.-laccase, 5.- hydrolyse of tyrosine, 6.- diaminooxidase, 7.- urease, 8.- gelatinase, 9.-caseinase, 10.- amylase

TABLE 1: RESULTS OF ENZYMATIC ACTIVITIES

Agaricales	ISOLATED	SYMBOL	1	2	3	4	5	6	7	8	9	10
Agaricus porphyrizon Orton	(1981)	AP 1	1	0	1	1	0	0	0	0	1	0
Agrocybe arenaria (Peck.) Sing.	(1983)	AA 2	0	1	0	0	1	1	1	0	1	0
A. dura (Bolt.: Fr.) Sing.	(1983)	AD 3	1	0	1	1	1	0	0	1	1	1
Armillaria borealis Marx. et Korh.	(1982)	AB 4	1	0	1	1	1	1	0	1	1	0
A. mellea (Vahl. in Fl. Dan.: Fr.) Karst.	(1983)	AM 5	1	0	1	1	1	0	0	1	1	0
Bolbitius vitellinus (Pers.: Fr.) Fr.	(1978)	BV 6	1	1	1	1	1	1	1	1	1	0
Calocybe constricta (Fr.) Kühn.	(1984)	CC 7	1	0	0	1	1	1	1	1	1	1
C. gambosa (Fr.) Sing.	(1981)	CG 8	1	0	1	1	1	1	1	0	1	0
- " -	(1986)		1	0	1	1	1	1	1	0	1	0
Clitocybe clavipes (Pers.: Fr.) Kumm.	(1982)	CC10	0	1	1	1	1	1	1	0	1	0
C. phyllophila (Fr.) Kumm. s. l.	(1982)	CC 9	1	1	1	1	1	1	0	1	1	0
Collybia dryophila (Bull.: Fr.) Kumm.	(1982)	CD11	0	0	0	1	1	1	1	1	1	0
C. peronata (Bolt.: Fr.) Kumm.	(1981)	CP12	0	1	0	0	1	0	1	0	1	0
Coprinus atramentarius (Bull.: Fr.) Fr.	(1983)	CA13	1	1	0	0	1	1	1	1	1	0
C. comatus (Müll. in Fl. Dan.: Fr.) Pers.	(1981)	CC14	1	0	1	1	1	0	0	0	1	0
- " -	(1982)		1	0	1	1	1	0	0	0	1	0
C. micaceus (Bull.: Fr.) Fr.	(1977)	CM15	1	0	0	1	1	1	1	1	1	1
Cystoderma terrei (Berk. et Br.) Harm.	(1984)	CT16	0	1	1	1	1	1	1	1	1	1
Flammulina fennae Bas		FF17	1	1	0	1	1	1	1	0	1	0
F. ononidis Arnolds	(1979)	FO18	0	0	0	0	1	1	1	0	1	0
- " -	(1981)		0	0	0	0	1	1	1	0	1	0
F. velutipes (Curt.: Fr.) Karst.	(1979)	FV19	0	0	0	1	1	1	1	0	1	0
Galerina marginata (Batsch) Kühn.	(1983)	GM20	0	1	1	1	1	0	0	0	1	1
Gymnopilus hybridus (Fr.: Fr.) Sing.	(1984)	GH21	1	0	1	1	1	1	0	1	1	0

Lacrymaria lacrymabunda (Bull.: Fr.) Pat.	(1983)	PV67	0	1	1	1	1	0	0	0	1	0
Lentinus lepideus (Fr.: Fr.) Fr.	(1983)	LL22	0	0	1	1	1	0	0	1	1	0
Lepista inversa (Scop.: Fr.) Pat.	(1981)	LI28	1	1	0	1	1	1	1	1	1	0
- " -	(1985)		1	1	0	1	1	1	1	1	1	0
L. irina (Fr.) Bigelow	(1984)	LI23	1	1	1	1	1	1	1	0	1	0
L. luscina (Fr.) Sing.	(1981)	LL24	0	0	1	1	1	1	1	1	1	0
- " -	(1984)		0	0	1	1	1	1	1	1	1	0
L. nebularis Batsch: Harm.	(1981)	LN25	1	0	1	1	1	1	1	1	1	0
L. nuda (Bull.: Fr.)Cke	(1981)	LN26	1	1	1	1	1	1	1	1	1	0
-"-	(1983)		1	1	1	1	1	1	1	1	1	0
-"-	(1982)		1	1	1	1	1	1	1	1	1	0
L. personata (Fr.: Fr.) Cke	(1984)	LS27	1	1	1	1	1	1	1	1	1	0
L. sordida (Schum.: Fr) Sing.	(1970)	LS29	1	1	1	1	1	1	1	1	1	0
Leucocoprinus bresadolae (Schulz.) Wass.	(1985)	LB30	1	0	1	1	1	0	0	0	1	0
L. denudatus (Rabh.) Sing.	(1981)	LD31	1	0	1	1	1	0	0	1	1	0
Leucoagaricus leucothites (Vitt.) Wass.	(1983)	LP32	1	1	1	1	1	0	0	1	1	0
Macrolepiota excoriata (Schff.: Fr.) Wass.	(1981)	ME33	0	0	1	1	1	0	0	1	1	0
M. rhacodes (Vitt.) Sing.	(1984)	MR34	1	0	1	1	1	0	0	0	1	0
Marasmius alliaceus (Jacq.: Fr) Fr.	(1975)	MA35	0	1	0	1	1	0	0	0	1	0
M. epiphyllus (Pers.: Fr.) Fr.	(1982)	ME36	0	1	0	0	1	1	1	0	1	0
M. oreades (Bolt.: Fr.) Fr.	(1981)	MO37	0	0	1	1	1	1	1	0	1	0
-"-	(1984)		0	0	1	1	1	1	1	0	1	0
M. scorodonius (Fr.: Fr.) Fr.	(1981)	MS38	1	0	1	1	1	0	1	0	1	0
Mycena abramsii Murr.	(1982)	MA39	1	0	1	1	0	0	1	0	1	0
M. crocata (Schrad.: Fr.) Kumm.	(1975)	MC40	1	0	0	0	1	1	1	0	1	0
M. galericulata (Scop.: Fr.) Quél.	(1983)	MG41	1	1	0	0	1	1	0	1	1	0
M. haematopus (Pers.: Fr.) Kumm.	(1977)	MH42	0	1	0	0	1	1	1	0	1	0
M. pseudopicta (Lge) Kühn.	(1981)	MP43	1	0	1	1	0	1	1	1	1	0

M. pura (Pers.: Fr.) Kumm.	(1982)	MP44	0	0	0	1	1	0	0	0	1	0
Oudemansiella mucida (Schrad.: Fr.) Höhn.	(1982)	OM45	1	1	1	1	1	1	1	1	1	0
O. radicata (Relhan: Fr.) Sing.	(1982)	OR46	1	1	0	1	1	1	1	1	1	0
Pholiota adiposa (Fr.) Kumm.	(1983)	PA47	1	1	0	1	1	1	1	0	1	0
P. alnicola (Fr.) Sing.	(1983)	PA48	1	0	0	0	1	0	0	0	1	0
P. carbonaria (Fr.) Sing.	(1986)	PC50	1	0	0	1	1	1	0	0	1	0
P. cerifera (Karst.) Karst.	(1984)	PA49	1	0	1	1	1	1	1	0	1	0
P. destruens (Brond.) Quél. s. l.	(1965)	PD51	0	0	1	1	0	1	0	0	1	0
-"-	(1983)		0	0	1	1	0	1	0	0	1	0
P. flammans (Fr.) Kumm.	(1982)	PF52	1	0	0	1	1	0	0	0	1	0
P. flavida (Schff.: Fr.) Sing.	(1980)	PF53	1	0	1	1	1	1	0	0	1	0
-"-	(1983)		1	0	1	1	1	1	0	0	1	0
P. gummosa (Lasch) Sing.	(1975)	PG54	1	0	1	1	1	1	1	0	1	0
-"-	(1982)		1	0	1	1	1	1	1	0	1	0
P. jahnii Tjall. et Bas	(1979)	PM57	1	0	1	1	1	1	1	0	1	0
P. lenta (Pers.: Fr.) Sing.	(1983)	PL55	1	0	1	1	1	0	1	1	1	0
P. lucifera (Lasch) Quél.	(1978)	PL56	1	0	0	1	1	1	1	1	1	0
P. spumosa (Fr.) Sing.	(1983)	PS58	1	1	1	1	1	1	1	1	1	0
P. squarrosa (Müll.: Fr.) Kumm.	(1985)	PS59	1	0	1	1	0	1	0	1	1	0
-"-	(1986)		1	0	1	1	0	1	0	1	1	0
Pleurotus dryinus (Pers.: Fr.) Kumm.	(1982)	PD61	1	1	1	1	1	0	1	1	1	0
P. eryngii (DC.: Fr.) Quél.	(1975)	PE62	1	1	0	1	1	1	0	0	1	0
-"-	(1981)		1	1	0	1	1	1	0	0	1	0
P. ostreatus (Jacq.: Fr.) Kumm.	(1984)	PO63	1	1	0	1	1	1	1	1	1	1
Pluteus romellii (Britz.) Sacc.	(1984)	PR64	0	1	0	1	1	1	1	1	1	1
Psathyrella candolleana (Fr.: Fr.) Maire	(1981)	PC66	1	1	0	1	1	1	1	1	1	0
P. prona (Fr.) Gill.	(1981)	PA65	0	0	0	1	1	1	1	1	1	0
Psilocybe cyanescens Wakefield	(1984)	PC68	1	1	1	1	1	1	0	0	1	1

P. semilanceata (Fr.) Kumm.	(1969)	PS69	0	1	1	1	1	1	1	1	1	0
Rhodocybe popinalis (Fr.) Sing.	(1981)	RP70	0	1	1	1	1	0	0	0	1	0
Stropharia coronilla (Bull.: Fr.) Quél.	(1981)	SC72	0	1	0	0	0	1	0	0	1	0
S. rugosoannulata Farlow: Murr.	(1983)	SR73	1	1	0	0	1	0	0	0	1	1
Aphyllophorales												
Ganoderma lipsiense (Batsch) Atk.	(1981)	GA74	1	1	0	0	1	1	1	0	1	0
Heterobasidion annosum (Fr.) Bref.	(1981)	HA75	1	1	0	1	1	1	1	0	1	0
Inonotus hispidus (Bull.: Fr.) Karst.	(1983)	IH76	0	1	1	1	1	0	0	1	1	0
Ischnoderma resinosum (Schrad.: Fr.) Karst.	(1981)	IR77	1	0	0	0	1	1	1	1	1	0
Laetiporus sulphureus (Bull.: Fr.) Murr.	(1982)	LS78	0	0	0	0	1	0	1	0	0	0
Meripilus giganteus(Pers.: Fr.) Karst.	(1983)	MG79	0	1	1	1	1	1	1	0	1	0
Merulius tremellosus Schrad.: Fr.	(1981)	MT80	1	1	1	1	1	1	0	1	1	0
Polyporus rhizophilus Pat.	(1978)	PR81	1	1	1	1	1	1	1	1	1	0
-''-	(1980)		1	1	1	1	1	1	1	1	1	0
-''	(1981)		1	1	1	1	1	1	1	1	1	0
P. squamosus (Huds.): Fr.	(1981)	PS82	1	0	1	1	1	0	1	1	1	0
Serpula lacrymans (Wulf.: Fr.) Schroet.	(1984)	SL83	0	0	1	1	1	1	1	0	0	0
Trametes hirsuta (Wulf.: Fr.) Pil.	(1982)	TH84	1	1	0	0	1	1	1	0	1	0
T. versicolor (L.) Pil.	(1982)	TV85	1	1	0	0	1	1	1	0	1	0
Gasteromycetes												
Bovista nigrescens Pers.: Pers.	(1982)	BN86	0	0	0	1	1	0	0	0	1	0
B. plumbea Pers.: Pers.	(1982)	BP87	1	0	1	1	1	0	0	1	1	0
B. pusilla (Batsch): Pers.	(1981)	BP88	1	0	1	1	1	0	0	1	1	0
Calvatia utriformis (Bull.: Pers.) Jaap	(1984)	CU89	1	0	0	1	1	0	1	0	1	0
Cyathus olla(Batsch): Pers.	(1984)	CO90	1	1	1	1	1	0	0	1	1	0
C. striatus (Huds.) Willd.	(1981)	CS91	1	1	0	1	1	0	0	1	1	0
Gastrosporium simplex Matt.	(1981)	GS92	1	0	0	0	1	1	1	1	1	0
Lycoperdon foetidum Bonord.	(1982)	LF93	1	0	1	1	0	1	0	1	1	0
L. lividum Pers.	(1984)	LL94	1	0	0	1	1	1	0	0	1	0

broad spectrum of enzymes including their quantitative determination in more strains or, occasionally, characterization of isoenzymes including their electrophoretic pattern.

The ability of individual cultures to preserve a certain enzyme activity for a longer time period was determined by repeatedly testing primary tyrosinase, amylases, pro-teinases and urease during 7 years. It could be shown that the absolute age of the cultures (i. e. the time that has passed since their isolation) has only a negligible effects on production of the enzyme studied. Some differences, quantitative differences in particular, can be caused by age of the colony on the Petri dish (i. e. by the relative age of the culture) as a result of a different growth rate. When testing a large number of cultures with a different growth rate it is difficult to investigate the whole studied collection during the identical growth phase. Most pronounced deviations were observed with urease. In general however, the enzyme activity is a highly stable marker and can thus be used as a useful characteristic feature of a species and as an auxiliary marker for classifi-cation of mycelial cultures of *Basidiomycotina*.

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