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ISOENZYME SPECTRA FOR CHARACTERIZATION AND IDENTIFICATION OF FUNGI (INFLUENCE OF GENETICAL AND NONGENETICAL FACTORS)

ISOENZYMSPEKTRA ZUR CHARAKTERISIERUNG UND IDENTIFIKATION VON PILZARTEN (EINFLUSS GENETISCHER UND NICHTGENETISCHER FAKTOREN)

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SUMMARY: Isoenzyme spectra for characterization, identification and delimitation of species can be quite valuable, especially where macroscopic and even microscopic characteristics are insufficient, provided proper precautions are taken. Such is the case with fungi. We investigated electrophoretical isoenzyme spectra of laccases. Our question was whether all the bands in the spectrum are determined genetically or whether the appearance of some bands might also depend on external and epigenetic factors. For this study we used two geographical races of the wood-rotting, edible basidiomycete *Pleurotus ostreatus*, the oyster mushroom. We traced the electrophoretic enzyme bands in the mycelium, the fruitbody and the culture filtrate through several generations and found that some bands were indeed dependent only on the part of the fungus examined or the composition of the medium. Others were always present and are therefore determined by genetical factors only. Thus they are suitable for characterization and identification of these species.

By comparing only the genetically stable laccase bands in some systematically problematic *Pleurotus* strains we found 2 groups of strains with similar or identical spectra, *P. ostreatus* and *P. columbinus* on the one hand, *P. eryngii, P. pulmonarius* and *P. cornucopiae* on the other hand, indicating identity or at least close relationship. This is in good agreement with the results of other authors using other methods. We also found that by crossing high-activity monokaryons, the resulting dikaryotic hybrid exhibited an even higher enzymatic activity. Furthermore, the ability to secrete specific laccase bands is

inherited and can be crossed into other strains. Both phenomena are of industrial importance.

Key words: Breeding, chemotaxonomy, electrophoresis, enzyme production, enzyme secretion, genetic control, industry, isoenzyme spectra, laccase, phenoloxidases.

Abbreviations: CF= culture filtrate, D = dikaryon, F1-generation = 1st filial generation, FB = fruitbody, IEF = isoelectric focusing, M = monokaryon, ME = mycelial extract.

RÉSUMÉ: L'analyse des spectres d'isoenzymes peut être un précieux auxiliaire pour la caractérisation, l'identification et la délimitation des espèces, si elle est utilisée de manière critique. Il en est ainsi des champignons dont les caractères macroscopiques et microscopiques sont insuffisants. Dans nos recherches des spectres d'isoenzymes de laccases par électrophorèse, nous avons d'abord vérifié si toutes les bandes du spectre sont déterminées génétiquement ou si la présence de quelques bandes peut dépendre de facteurs externes ou épigénétiques. Pour ce faire, deux races géographiques de *Pleurotus ostreatus* ont été examinées. Les laccases du mycélium, du basidiome et du filtrat des cultures furent analysées. Le résultat montre que la présence de quelques bandes dépend de la partie du champignon considérée ou de la composition du milieu de culture. D'autres bandes en revanche sont toujours présentes, elles sont génétiquement déterminées et sont utilisables pour la caractérisation, l'identification et la délimitation des espèces.

La comparaison des bandes de laccase génétiquement stables de quelques souches de *Pleurotus* problématiques du point de vue systématique a mis en évidence 2 groupes à spectres d'isoenzymes différents: *P. ostreatus* et *P. columbinus* d'une part, *P. eryngii, P. pulmonarius* et *P. cornucopiae* d'autre part. Ceci prouve l'identité ou du moins la proche parenté des espèces au sein de chaque groupe. Ce résultat correspond d'ailleurs bien à ceux obtenus par d'autres auteurs utilisant des méthodes différentes. En croisant des monocaryons à forte activité enzymatique, on peut obtenir des hybrides dicaryotiques à production enzymatique encore accentuée. De plus, la faculté de sécréter des laccases spécifiques est transmissible et peut être introduite dans des souches par croisements. Ces deux phénomènes sont susceptibles de revêtir une grande importance dans l'industrie.

Mots clés: Culture, chimiotaxonomie, électrophorèse, production enzymatique, sécrétion enzymatique, contrôle génétique, industrie, spectre d'isoenzymes, laccases, phénoloxydases.

ZUSAMMENFASSUNG: Isoenzymspektra können wertvolle Hilfsmittel bei der Charakterisierung, Identifizierung und Artabgrenzung sein, wenn die Methode kritisch angewandt wird. Dies ist insbesondere bei Pilzen der Fall, wenn makroskopische oder sogar mikroskopische Daten unzureichend sind. Bei der vorliegenden Untersuchung elektrophoretischer Isoenzymspektren der Laccase wurde zunächst geprüft, ob alle Banden des Spektrums genetisch determiniert sind, und ob das Auftreten einiger Banden auch von externen und epigenetischen Faktoren abhängen kann. Dazu wurden zwei geographische Rassen des holzabbauenden, essbaren Basidiomyceten *Pleurotus ostreatus*, des Austernseitlings, eingesetzt. Laccasen aus dem Myzel, dem Fruchtkörper und dem Kulturfiltrat wurden untersucht. Es zeigte sich, dass das Auftreten einiger Banden von dem untersuchten Teil des Pilzes oder der Medienzusammensetzung abhängig war. Andere Banden dagegen traten immer

auf, sie sind nur genetisch determiniert und daher allein zur Charakterisierung, Identifizierung und Artabgrenzung verwendbar.

Durch einen Vergleich nur der genetisch stabilen Laccasebanden von einigen systematisch problematischen *Pleurotus*-Stämmen wurden zwei Gruppen mit ähnlichen oder identischen Isoenzymspektren gefunden: *P. ostreatus* und *P. columbinus* einerseits, *P. eryngii, P. pulmonarius* und *P. cornucopiae* andererseits. Dies deutet auf Identität oder zumindest nahe Verwandtschaft innerhalb der Gruppe hin. Das Ergebnis steht in guter Übereinstimmung mit den mit anderen Methoden erhobenen Befunden anderer Autoren. Dass durch Kreuzung Laccase-hochaktiver Monokaryen die resultierenden dikaryotischen Hybriden eine nochmals gesteigerte Enzymproduktion aufweisen und dass die Fähigkeit, spezifische Laccase-Isoenzyme auszuscheiden vererbbar ist und in andere Stämme eingekreuzt werden kann, ist möglicherweise von hoher industrieller Bedeutung.

Schlüsselwörter: Züchtung, Chemotaxonomie, Elektrophorese, Enzymproduktion, Enzymausscheidung, genetische Kontrolle, Industrie, Isoenzymspektren, Laccase, Phenoloxidasen.

I. INTRODUCTION

Unequivocal identification and establishment of systematic relationships in fungi is often problematic, in particular if macro-properties such as fruitbodies are lacking or even micro-properties are insufficient. On the other hand fungi are gaining increasing importance in industry as food and feed and for the production of pharmaceuticals and other metabolic products. Here in particular, correct identification e.g. for patenting of strains is very important.

Also for chemotaxonomical questions in fungi such as delimitation and characterization of species, physiological and biochemical criteria are becoming more and more important. For this purpose electrophoretical enzyme spectra have been used increasingly (BLAICH, 1977; BRESINSKY, 1977; PRILLINGER & MOLITORIS, 1979; ROYSE & MAY, 1982). In the preceding paper (PRILLINGER & MOLITORIS, 1979) in particular, the problem of microevolution (speciation) in fungi has been investigated by following the variation and inheritance of individual enzymes in electrophoretic enzyme spectra. The present paper is the result of further work along these lines.

When using isoenzyme spectra in chemotaxonomy, the results are, however, not always satisfying since isoenzyme spectra in fungi - although reproducible and characteristic - can differ not only within a family, but also within a given species or even strain (MOLITORIS, 1979; PRILLINGER & MOLITORIS, 1979). Apparently not only genetic factors, but also non-genetic, external and epigenetic factors might be responsible

for an isoenzyme spectrum of a given fungal strain. Caution in interpretation is therefore necessary (BRESINSKY, 1977; HURKA, 1980).

In order to test the influence of various factors on isoenzyme spectra we investigated the zymograms after growth on different substrates and at different developmental stages of the fungus (mycelium, fruitbody, culture filtrate). To include the often overlooked genetic aspect we also observed the spectra of mono- and dikaryotic material over several generations. *P. ostreatus* was chosen because this fungus has been investigated morphologically and genetically quite well (BRESINKSY & al., 1979; FISCHER, 1981; HILBER, 1982; PAULUS, 1985), because it is widely cultivated as an edible mushroom and because this genus, in addition, produces a number of antibiotics (ANKE, 1978; MOLITORIS, 1979).

Phenoloxidases, in particular laccase (EC 1.10.3.2), were chosen as test enzymes for the following reasons: Phenoloxidases were among the first enzymes used for ecological characterization of fungi as brown rot and white rot fungi (BAVENDAMM, 1928; LYR, 1958). Phenoloxidases are involved in wood degradation and are therefore ecologically very important (ANDER & ERIKSSON, 1976; MOLITORIS, 1979; VETTER, 1985) and in the future may play an important role in the lumber and paper industry. Phenoloxidases have been shown to give reliable multienzyme spectra (MOLITORIS, 1979; PRILLINGER & MOLITORIS, 1979).

II. MATERIALS AND METHODS

1. Strains: *Pleurotus ostreatus* (Jacq. ex Fr.) Kummer. Strain 1s: BRD, near Münster, *Fagus silvatica*, leg. et det. Runge. Strain 1w: Japan, not *Fagus*, commercial strain 4612, Mori Mushroom Res. Inst., Japan. Strain 1a: BRD, Karlsruhe, *Juglans*, leg. Grinbergs, det. Hilber. *P. columbinus* Quél. Strain 3d: BRD, Grünstadt-Asselheim, *Eryngium campestre*, leg. et det. Winterhoff. Strain 3e: CSSR, Branany near Bilinar, *Eryngium campestre*, leg. et det. Klan. P. *pulmonarius* (Fr.) Quél. Strain 4i: BRD, Zwieseler Waldhaus, Böhmerwald, *Fagus*, leg. et det. Hilber. *P. cornucopiae* (Paulet ex Pers.) Rolland. Strain 4R: CSSR, Cahnov near Lanzhot, *Ulmus*, leg. et det. Pouzar. All monokaryons were checked microscopically for lack of clamp connections.

2. Media: Glucose-peptone medium (HASHIMOTO and TAKAHASHI, 1976). Sterilization of media 15 min at 121°C. For data on production of

fruitbodies (culture conditions), harvesting mycelia, preparation of mycelial extracts and culture filtrates see BRESINSKY & al. (1977).

3. Electrophoreses: Disc electrophoresis. pH 4.3 in 7.5% polyacrylamid gel (REISFELD & al., 1962). Further details in PRILLINGER and MOLITORIS, (1979). Slab gel electrophoresis. DESAGA PAG screening electrophoresis system HAVANNA; method according to ORNSTEIN and DAVIS (1964, modified: separation gel pH 9.0, 10% acrylamide; instead of the concentration gel a separation gel with only 5% acrylamide was used). Sample volume 30 µl. Isoelectric focusing in polyacrylamide gel (WRIGLEY, 1968). Acrylamide 7.5%, methylene-bis-acrylamide 0.2%, ampholine LKB pH 3-10 or pH 3.5-5.0. Further details in PRILLINGER and MOLITORIS, (1979).

4. Laccase activity: Presence of laccase in mycelial extracts or culture filtrates was determined colorimetrically at 468 nm with 0.65 mM 2.6-dimethoxiphenol in 0.1 M Na-citrate/NaOH buffer, pH 5.0, according to PRILLINGER (1976). 1 unit of laccase activity (1 U) corresponds to a difference in absorption of 0.2/min at 468 nm and 1 cm light path. Activity is based on dry weight of mycelium (for culture filtrates: on the amount of mycelial dry weight present in the culture producing a certain amount of culture filtrate). Staining for laccases after electrophoresis and isoelectric focusing with benzidine (BRESINSKY & al., 1977). All enzymatic procedures except electrophoreses (room temperature) were performed at 0 - 4° C.

III. RESULTS

1. Fruitbodies and mono- and dikaryotic mycelia. Fruitbodies (FB, 1s, 1w) were grown from the dikaryotic mycelium of the two geographical races 1s and 1w of *Pleurotus ostreatus* (fig. 1). Monokaryotic cultures (Parental generation, 1s1 to 1s20, 1w1 to 1w20) were obtained from randomly isolated basidiospores of each fruitbody. By confrontations of these monokaryotic mycelia on agar plates a number of dicaryotic hybrids (e.g. 1s4 x 1w8) were produced from which fruitbodies (1s4 x 1w8) were grown. From single basidiospores of these fruitbodies the monokaryotic cultures "a" to "h" of the F1-generation were obtained. Extracts were prepared from fruitbodies (FB) and from mycelia (ME) and were investigated together with the culture filtrates (CF) for laccase activity and electrophoretic isoenzyme spectra.

2. Breeding for increased enzyme activity. Generally, the laccase activity (U/g dry weight) was lowest in fruitbodies and highest in

culture filtrates. Table 1 shows the extracellular laccase activity of the natural dikaryons, of several monokaryons of the parental generation and of some dikaryotic hybrids. Generally, the laccase activity in dikaryotic material was higher than in monokaryotic material. The monokaryotic progeny shows a high variability in extracellular laccase activity. Crossing the two monokaryons with the highest activity (1s23: 448; 1w11:235), results in a dikaryon (1s23 x 1w11) with an activity of 618 which is well above the activity of the component monokaryons (235, 448) and above the calculated average activity of the dikaryon (342). An increase in enzyme production (and secretion) by crossing of high-producing strains seems therefore possible.

3. Electrophoresis and isoenzyme pattern. Although a complex malt broth medium gave higher enzyme production, a semisynthetic glucose-peptone medium was used for growing *P. ostreatus* when it was found that the reproducibility of the laccase isoenzyme spectrum was much better using the latter medium. In addition, it had been observed in preceding experiments that a number of laccase bands in the region of Rf 0 - 0.2 after flat gel electrophoresis at pH 9.0 showed poor reproducibility, and these bands were therefore excluded from further consideration in this context.

As shown in figure 2, 7 distinct and well reproducible laccase isoenzymes could be found by using flat gel electrophoresis at pH 9.0, disc electrophoresis at pH 4.3 and isoelectric focusing. Afterwards we investigated which factors influence synthesis and secretion of these laccases qualitatively and quantitatively.

4. Selective, genetically controlled laccase secretion. Figure 3 shows diagrammatically in the top row the presence and strength of laccase isoenzymes 1 and 2 for mycelial extracts (ME), in the bottom row for culture filtrates (CF), both for representative strains of the generations tested (from natural isolate on the left over a hybrid dikaryon to the F1-generation on the right).

In the two natural isolates 1s and 1w and in the monokaryotic strains (1s4, 1w8) of the parental generation shown, both enzymes are produced intracellularily but only one is secreted (laccase 1 by the s-strains, laccase 2 by the w-strains).

Following the enzyme spectra of mycelial extracts and culture filtrates from left to right indicates (together with the results on further strains not shown in this figure) that intracellular synthesis and secretion is genetically controlled individually for both laccase 1 and 2. This is evident in particular for the secretion process, where in the culture filtrate of the dikaryon 1s4 x 1w8 both laccases, 1 and 2, are secreted. In

the monokaryotic offspring (F1-generation) an approximate 1:1 (5:3 out of 8 tested) segregation for the parental types is found. It is therefore possible to breed not only for intracellular synthesis of individual laccase isoenzymes but also for secretion of specific laccases into the culture filtrate.

5. Specificity of laccase isoenzymes. One of the main questions in this project was to see whether *all* of the multiple enzyme bands of the spectra of a given species are equally influenced by genetic, epigenetic and environmental factors and are therefore all equally suited for chemotaxonomic purposes, as is commonly assumed and often practised. If, however, individual enzyme bands react individually to these factors, a much more detailed knowledge of the intraracial and intraspecies variablity of such spectra would be required in order to make chemotaxonomically relevant statements.

The following describes which laccase isoenzymes depend for their synthesis on which factors. Figure 4 shows from an electrophoresis of a mycelial extract that laccase 7 is substrate-dependent, because it is found only after growth on wheat grain medium. In addition, this isoenzyme is also dependent on the developmental stage since it appears in mycelium only. In contrast to this, laccase 6 is found exclusively in fruitbodies, irrespective of the medium used.

Table 2 summarizes the results obtained for the specificity of the laccase isoenzymes. In addition to the laccase isoenzymes 1 and 2, shown in figure 3 and laccases 6 and 7, shown in figure 4, it was found that laccases 3 and 4 are also dependent on the developmental stage inasmuch as they increase in activity with time of growth.

Isoenzyme 5 apparently is a mutatively altered protein, since it appeared after meiosis only and in only one out of 48 monokaryons tested.

Evaluating the presence and the behaviour of laccases 1 through 7 in the isoenzyme spectra through the generations investigated, only laccases 1, 2, 3 and 4 are exclusively genetically controlled and show no variation. Only these isoenzymes should therefore be used for chemotaxonomical purposes.

6. Chemotaxonomy of *Pleurotus* species using laccase spectra. The *Pleurotus*-complex is taxonomically rather problematic, since by just using morphological characters species delimitation is still controversial here.

Preparing extracts from fruitbodies of several strains of *P. ostreatus*, *P. columbinus*, *P. eryngii*, *P. pulmonarius* and *P. cornucipiae* and subjecting them to flatgel electrophoresis, pH 9.0, laccase spectra were

obtained. If only the Rf region is considered where in *P. ostreatus* the chemotaxonomically relevant laccase isoenzymes 1 and 2 are found, two groups of *Pleurotus* strains can be differentiated as shown in figure 5.

One group comprises *P. ostreatus* and *P. columbinus*, both showing identical spectra with laccase 1 *and* laccase 2. The other group with *P. eryngii, P. pulmonarius* and *P. cornucopiae* is different, showing only band 2 and/or an additional band "x". Since here mycelial extracts were investigated, the secretion process (see under "4. Selective secretion") cannot be responsible for this result. The data therefore indicate a close relationship or even identity of *P. ostratus* and *P. columbinus*, different from the other species investigated. Recent results of other authors using different methods are in good agreement with this finding.

IV. DISCUSSION

Fungi, in particular basidiomycetes, have so far been discussed relatively infrequently in chemotaxonomic literature in contrast to bacteria and higher plants (for references see PRILLINGER & MOLITORIS, 1979; HEYWOOD & MOORE, 1984). This was one of the reasons for choosing the basidiomycete *Pleurotus ostreatus* for this study, which in the beginning dealt primarily with the process of speciation in fungi. Since in the relatively few papers on higher basidiomycetes the genetic aspect is more or less overlooked and since mostly dikaryotic strains have so far been investigated, our study observed enzyme production and inheritance of isoenzymes over several generations of mono- and dikaryotic material.

It has previously been shown that phenoloxidases, in particular laccase, can be induced by specific substrates (FROEHNER & ERIKSSON, 1974a; LEONOWICZ & TROJANOSWSKI, 1975a, b; MOLITORIS, 1979) and that laccase synthesis is controlled genetically (FROEHNER & ERIKSSON, 1974a, b; PRILLINGER & ESSER, 1977). In this paper (and also in PRILLINGER & MOLITORIS, 1979) is shown that specific laccase isoenzymes are controlled in synthesis *and* secretion by separate structural genes. Since phenoloxidases are involved in several steps of wood degradation (lignin and celluose), it is of considerable interest that laccase synthesis and secretion can be crossed into other compatible strains and increased in amount by breeding.

Participation of phenoloxidases in fungal morphogenesis has been postulated and shown to occur in several cases (for references see MOLITORIS, 1976; also HIRSCH, 1954; PHILLIPS & LEONARD, 1976;

PRILLINGER & ESSER, 1977). The fact that certain laccase isoenzymes in the present study were found exclusively in specific developmental stages (ME, FB) points in the same direction.

In the following discussion of the chemotaxonomical aspects comparing the laccase isoenzyme spectra of different *Pleurotus* species, we shall exclude the so-called *Pleurotus ostreatus* "Florida" strains because their identity is questioned and the results on their natural relationship are very controversial (BRESINSKY & al., 1977; EGER & al., 1977; HILBER, 1982).

Several strains of *Pleurotus columbinus, P. cornucopiae, P. eryngii, P. ostreatus* and *P. pulmonarius* were compared on the basis of their laccase isoenzyme spectra. In particular the laccases 1 and 2 could be shown to be genetically stable and possibly suitable for chemotaxonomic purposes. The results obtained suggest that *P. columbinus* and P. *ostreatus* (both containing laccase 1 and 2) are identical or at least closely related, whereas *P. cornucopiae, P. eryngii* and *P. pulmonarius* are different. The latter strains show also differences within the group by presence of only laccase 2 and/or an additional laccase "x".

These findings have been confirmed in full or in part for all or at least for some of the strains investigated here by other authors using different methods. Indentity or at least close relationship between *P. ostreatus* and *P. columbinus* was found by EGER & al. (1977) by using strain intercompatibility as a criterion (however, EGER & al. (1977) found also compatibility of *P. ostreatus* with *P. pulmonarius* and *P. sapidus*). The same identity or close relationship observed MANNING (1977) using formation of clamp connections as indicator (they were formed between *European* strains of *P. ostreatus* and *P. columbinus* or *P. sapidus*, respectively, they were *not* formed between *American* strains of *P. ostreatus* and *P. columbinus* or P. *sapidus*, respectively).
Differences between P. *ostreatus* on the one hand and *P. cornucopiae, P. eryngii* and *P. pulmonarius* on the other hand using compatibility as the criterion, were recently found by BRESINSKY & al.(1977), HILBER (1977a, b), and FISCHER (1981).

PAULUS (1985) found that none of the *P. ostreatus* strains of different origin that he investigated was compatible with *P.pulmonarius*.

The fact that *P. eryngii* and *P. cornucopiae* represent good, distinct species of their own and are not compatible with each other and any one of the other species investigated in the respective studies was recently found by a number of authors (BRESINSKY & al., 1977; HILBER, 1977a, b; FISCHER, 1981).

In summary, we were able to show that it is problematic to use isoenzyme spectra uncritically for chemotaxonomic purposes unless proper precautions are taken, such as using comparable and constant conditions for cultivation and preparation of material and excluding from consideration enzyme bands which are not species-specific but rather depend on other factors. Therefore, normally the classical morphological, microscopical (and perhaps genetical) methods should be used first. In cases where these characteristics are lacking or insufficient (see also HURKA, 1980), however, isoenzyme spectra could be used with advantage, not only for chemotaxonomic purposes such as species delimitation, but also for identifying and patenting fungal strains in industry.

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Table 1: Breeding for maximal activity of extracellular laccase in *Pleurotus ostreatus*.
Activity of extracellular laccase of the dikaryotic geographical races 1s and 1w, their monokaryotic progeny and some dikaryotic hybrids. Activity is expressed in U/g dry weight (see material and methods). The average of at least three determinations is given. For origin of strains see figure 1.

Original dikaryons (D)	**Monokaryons (M)** (from basidiospores)	**Hybrids (D)**			
Strain / Activity (U/g dry weight)	Strain / Activity (U/g dry weight)	Strain / Activity (U/g dry weight) component monokaryon		dikaryon calculated	dikaryon measured
1s (BRD) 553	1s4 189	1s4	189	189	240
	1s17 128	×			
	1s18 319	1w8	189		
	1s22 186				
	1s23 448	1s17	128	182	279
	1s25 60	×			
		1w11	**235**		
1w (Japan) 746	1w8 189	1s22	186	211	169
	1w9 184	×			
	1w11 235	**1w11**	**235**		
	1w12 121	**1s23**	**448**	**342**	**618**
	1w15 69	×			
	1w16 204	**1w11**	**235**		
		1s23	**448**	285	278
		×			
		1w12	121		

Table 2: Specificity and properties of laccase isoenzymes of *Pleurotus ostreatus*.
A summary is given for environmental, genetical and epigenetical factors responsible for synthesis and secretion of laccase isoenzymes 1 to 7. As a result, the isoenzyme bands suitable for chemotaxonomical investigations are listed.

Property / Dependence		Isoenzyme band number						
Dependent on		1	2	3	4	5	6	7
Substrate								+
Development	mycelial extract only							+
	fruitbody only						+	
	culture filtrate (spec. secretion)	+	+					
	quantitatively variable			+	+			
Other	mutatively altered					+		
	genetically stable	+	+	(+)	+			
Chemotaxonomically usable		+	+	+	+			

Fig.1. Origin and genealogy of the mono- and dikaryotic strains of *Pleurotus ostreatus* tested for laccase isoenzymes by electrophoresis.

Starting with two dikaryotic geographical races (natural isolates 1s, 1w), fruitbodies were produced (1s, 1w), the basidiospores of which gave rise to monokaryotic cultures (1s1 to 1s20, 1w1 to 1w20) of the parental generation. Several of these monokaryotic cultures were crossed, resulting in dikaryotic hybrids (e.g. 1s4 x 1w8) from which fruitbodies were obtained (e.g. 1s4 x 1w8). The monokaryotic mycelia of the F1-generation ("a" to "h") were obtained from single basidiospores of these fruitbodies.

Fig. 2. Electrophoretic methods and the resulting isoenzyme spectra of laccases of *Pleurotus ostreatus*.

The banding pattern and Rf-values are given for laccase isoenzymes 1 through 7 after flatgel electrophoresis (pH 9.0) and disc electrophoresis (pH 4.3). The IEP is given for the laccase isoenzymes 1 and 2 after isoelectric focusing in the range pH 4.0 to 5.0. For further details see material and methods.

Fig. 3. Evidence of genetic control of synthesis and secretion of laccase isoenzymes by electrophoresis of intracellular and extracellular laccases through several generations of *Pleurotus* mycelium.

The (vertical) differences between the isoenzyme pattern of mycelial extracts (top) and culture filtrates (bottom) indicate secretion of the respective isoenzymes into the medium. The (horizontal) differences within the mycelial extracts (top) and within the culture filtrates (bottom) through the generations tested (from left to right) indicate genetic control of individual isoenzymes. The appearance of both laccases, 1 and 2, in the culture filtrate of the dikaryotic parental generation (1s4 x 1w8) points to a selective, genetically controlled secretion. This genetic control is also indicated in the culture filtrate by segregation with an approximate 1:1 (5:3 out of 8) ratio of the parental types in the F1-generation. Slab gel electrophoresis, pH 9.0; anode at the bottom. For further details see text.

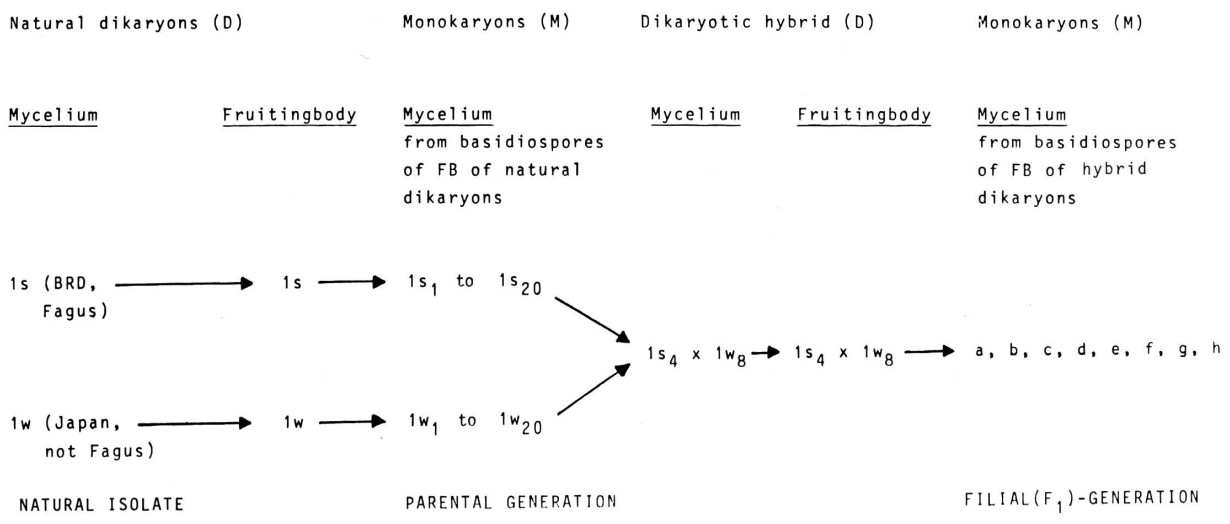
Natural dikaryons (D)
Monokaryons (M)
Dikaryotic hybrid (D)
Monokaryons (M)
Mycelium
Fruitingbody
Mycelium from basidiospores of FB of natural dikaryons
Mycelium
Fruitingbody
Mycelium from basidiospores of FB of hybrid dikaryons
1s (BRD, Fagus)
1s
1s1 to 1s20
1w (Japan, not Fagus)
1w
1w1 to 1w20
1s4 x 1w8
1s4 x 1w8
a, b, c, d, e, f, g, h
NATURAL ISOLATE
PARENTAL GENERATION
FILIAL(F1)-GENERATION

Fig.1.

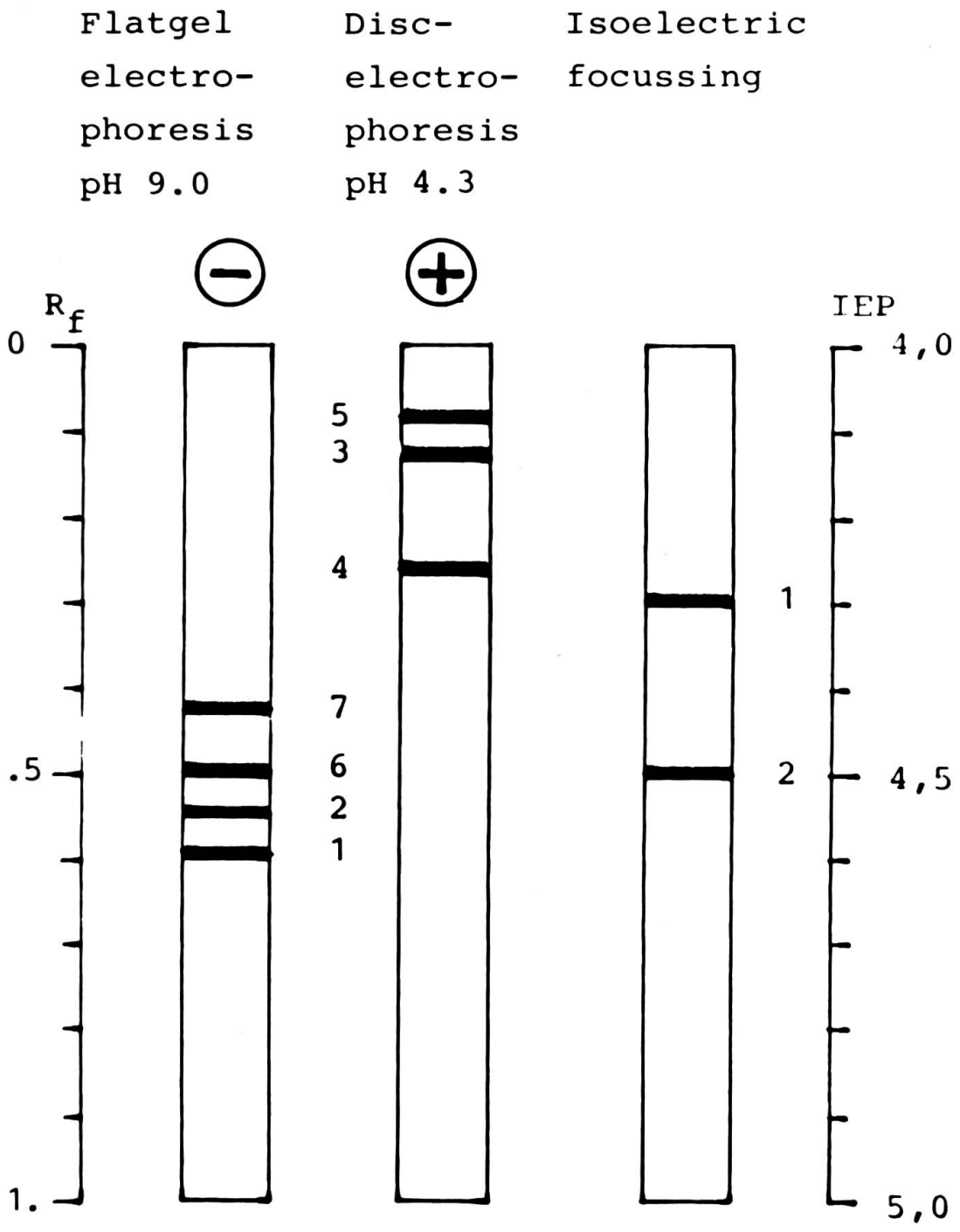
Flatgel
electro-
phoresis
pH 9.0
Disc-
electro-
phoresis
pH 4.3
Isoelectric
focussing
Rf
0
.5
1.
5
3
4
7
6
2
1
1
2
IEP
4,0
4,5
5,0

Fig. 2.

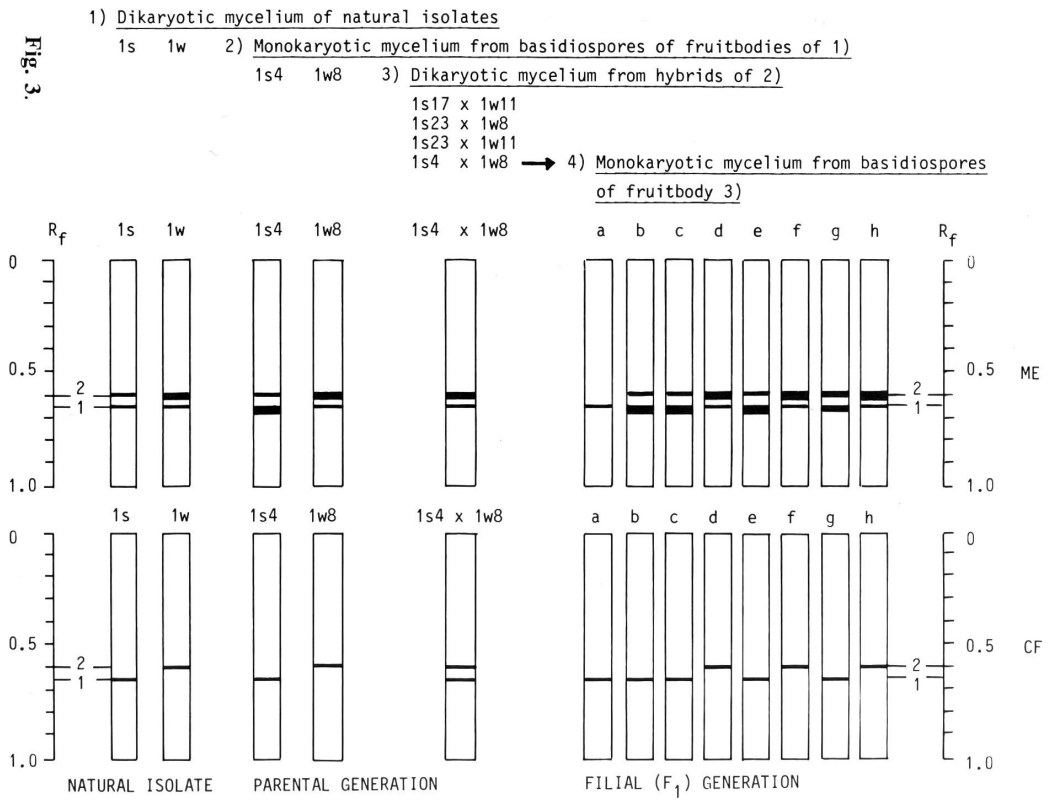
1) Dikaryotic mycelium of natural isolates
1s 1w
2) Monokaryotic mycelium from basidiospores of fruitbodies of 1)
1s4 1w8
3) Dikaryotic mycelium from hybrids of 2)
1s17 x 1w11
1s23 x 1w8
1s23 x 1w11
1s4 x 1w8
4) Monokaryotic mycelium from basidiospores of fruitbody 3)
Rf
0
0.5
1.0
2
1
ME
CF
a b c d e f g h
NATURAL ISOLATE
PARENTAL GENERATION
FILIAL (F1) GENERATION

Fig. 3.

Fig. 4. Evidence of development and substrate-specific laccase isoenzymes of *Pleurotus ostreatus*.

Mycelia and fruitbodies of the two geographical races 1s and 1w and of the hybrid 1s4 x 1w8 and of their monokaryotic progeny were grown on different media. After electrophoresis of the respective extracts, laccase isoenzyme 7 appeared only after growth on wheat grain medium (this figure) and only in mycelial extracts; this band therefore is substrate and development-specific. Laccase isoenzyme 6 is only detected in fruitbodies, whereas isoenzymes 1 and 2 are always found independently from substrate and developmental stage. Slab gel electrophoresis, pH 9.0; anode at the bottom.

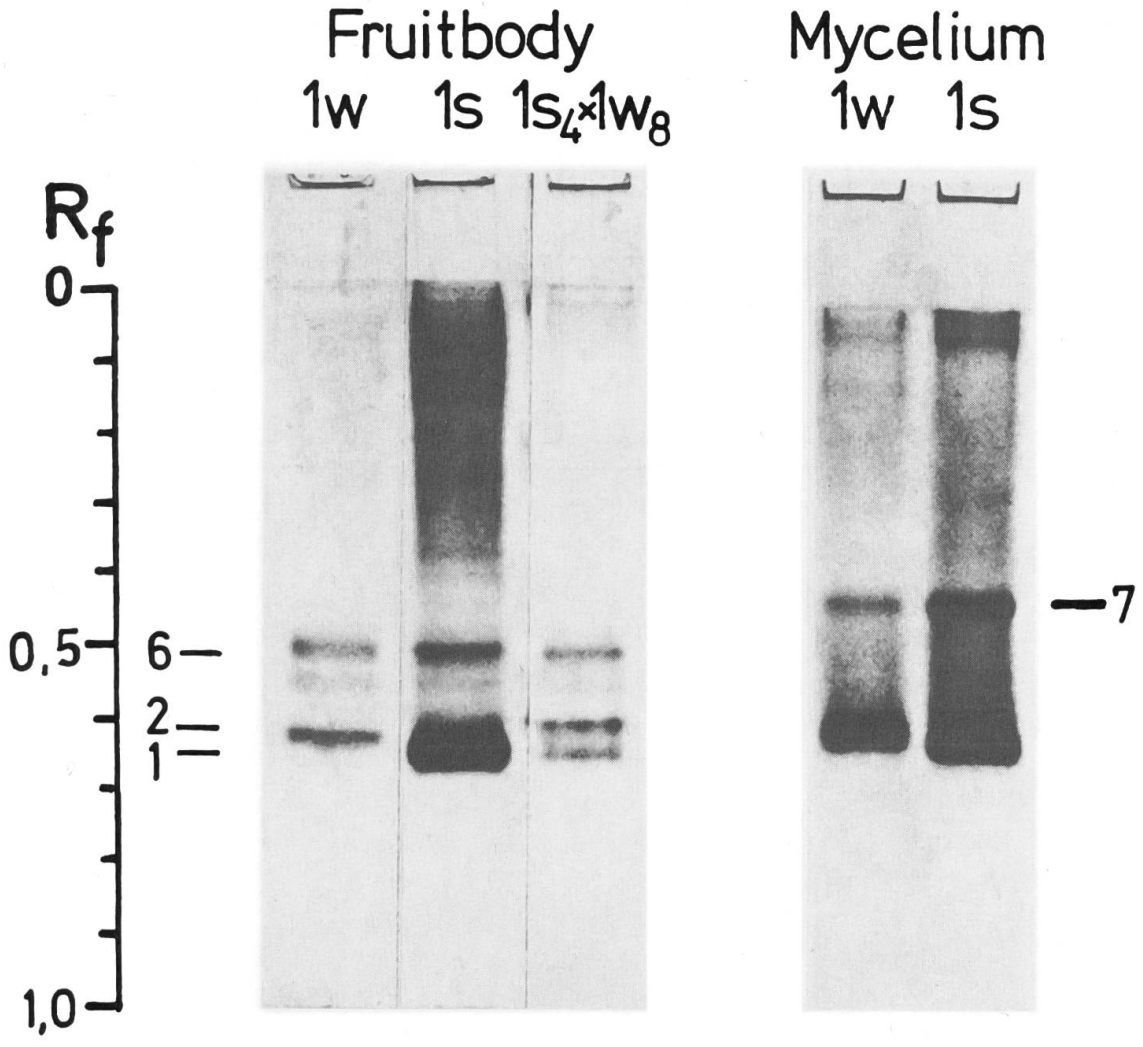

Fig. 5. Laccase isoenzyme spectra of different species of *Pleurotus*. Growth of dikaryotic mycelia on glucose-peptone medium for 14 days at 27° C. Flatgel electrophoresis of mycelial extracts at pH 9.0; anode at the bottom. For further details see material and methods and text.