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Genetic Analysis in Wood-Decaying Fungi

Genetic Variation and Evidence for Allopatric Speciation in *Pleurotus ostreatus* using Phenoloxidase Zymograms and Morphological Criteria

By

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Abstract

Using electropherograms (zymograms) of the phenoloxidase laccase and characteristics of mycelial growth and fruit body production, a distinct morphological and biochemical differentiation of two geographically isolated (allopatric) populations of the wood-rotting basidiomycete *Pleurotus ostreatus* became evident. No limitation in their outbreeding ability was observed, however. A specific secretory mechanism for an extracellular laccase, genetically different in the two geographical races, could be detected. An approximately 1 : 1 segregation of this laccase band in the F_1 generation indicates that specific secretion of this enzyme is controlled by one gene only. Different degrees of genetic variation as shown by differences in the respective laccase spectra were found in the two geographical races. Only one enzyme band out of nine multiple laccases was found to be specific for fruit bodies. The value of zymograms for chemotaxonomic purposes, for the understanding of microevolution and for determination of genetic variation in fungi is critically discussed.

Keywords: Chemotaxonomy, developmental variation, genetic variation, isoenzymes, laccase, Pleurotus, speciation, wood-rotting, zymograms.

Introduction

Since Markert and Moller (1959) first detected that isoenzymes are very common in organisms, a rapidly increasing number of publications dealing with this problem has appeared. Although the term *'isoenzyme'* is commonly used in its broadest sense, including all enzymes with similar or identical catalytic activity, the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (IUPAC-IUB 1978) should not be overlooked. The use of the term *'multiple forms of the enzyme'* is recommended to refer to all proteins catalysing the same reaction and occurring naturally in a single species, whereas *'isoenzyme'* should apply only to those enzymes arising from genetically determined differences in the primary structure excluding only modified enzymes.

For the higher fungi, there is much information on the comparison of electrophoretic zymograms (electropherograms, enzyme spectra) as an additional taxonomic tool for a clear delimitation of species (for references see Blaich 1977). On the other hand Bresinsky (1977) has pointed out the risk of overestimating the above-mentioned criteria for use in separating species as long as little is known about the genetic variation of species. He has further referred to a more valuable role for zymograms in the understanding of microevolution by considering the possibility of detecting genetic variation within a species using this method. Especially in plants it is known since Turesson (1922, 1925, 1930) that

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genetic variability is correlated with ecological properties (*e.g.* soil, climate). Ayala (1975) has stressed the usefulness of isoenzymes in detecting the amount of genetic differentiation in the process of speciation, and has reviewed the literature on *Drosophila* where allopatric speciation was detected in the *Drosophila willistoni* group.

Allopatric speciation is the term used to denote the differentiation of geographically isolated (allopatric) populations to the point where they are considered to be taxonomically a discrete species, whereas in sympatric speciation this process occurs in the absence of geographic isolation (Rieger *et al.* 1976). Two different processes become apparent during speciation: 1. Differentiation within different populations; 2. Genesis of reproductive isolation. Using isoenzymes, genetic variability was found to increase long before sexual propagation was blocked. In contrast to this, it is known that genetic isolation preventing sexual propagation in the ascomycetes can occur before morphological and physiological differentiation can be observed (Rizet 1952, Esser 1959, Esser and Blaich 1973). In higher basidiomycetes little is known about speciation. Kemp's (1975, 1977) studies on species of *Coprinus* suggest that sympatric speciation at the cellular level may be common in basidiomycetes.

For our investigations we have chosen the section of *Pleurotus* (according to Singer 1975) because these fungi occur around the world on quite different substrates (deciduous trees and conifers) and produce fruit bodies within an acceptable time in the laboratory. In order to show genetic variation by using a multiple enzyme system, phenoloxidases, especially laccase (EC 1.10.3.2) were used because they are widespread in fungi, participate in wood and lignin degradation, and occur in multiple forms (for literature see Molitoris 1976, 1978). Molitoris (in Bresinsky *et al.* 1977) gives some evidence for qualitatively altered zymograms in two geographical races of *Pleurotus ostreatus* by analysing multiple forms of the phenoloxidase laccase.

We, therefore, were interested to see whether these differences represent only *genetic variation* within the monokaryotic isolates of a single wild strain or whether they indicate *genetic differentiation* leading to the formation of distinct species. In the latter case they are useful as diagnostic tools to characterize geographical races of a given species.

Abbreviations: CF, culture filtrate; D, dikaryon; DMOP, 2,6-dimethoxyphenol; d.w., dry weight; F,, 1st filial generation; FB fruit body; IEP, isoelectric point; M, monokaryon; ME, mycelial extract; SE, standard error; U, units laccase activity.

Materials and Methods

Strains

Geographical races of *Pleurotus ostreatus* (wild strains): Is leg. A. Runge on *Fagus sylvatica* near Munster (BRD), isolated by O. Hilber. lw was obtained from K. Mori. It is only known that the natural substrate of lw is different from *Fagus* (Mori, unpublished, Mori Mushroom Research Inst., Hiraicho, Kiryu, Japan; No. 4612). For monokaryons ls, and lw, and the dikaryotic hybrid see Bresinsky *et al.* 1977. All the further monokaryons were obtained by a colonial growth method on sorbose yeast extract medium (Esser and Prillinger 1972). On this medium the germination rate of I' basidiospores was between 16-38%, for lw basidiospores 0.2-16%. All monokaryons were checked microscopically for lack of clamp connections.

Media

1.5% malt-broth; glucose-peptone medium (Hashimoto and Takahashi 1976); synthetic medium: $NH_4H_2PO_4$ I'-4 M, L-valine 8.5 mM, sucrose 58.4 mM, asparagine 6.7 K_2HPO_4 x $3H_20$ 0.98 mM, KH_2PO_4 5.9 mM, $MgSO_4$ x 7 H_20 3.6 mM, $CaCl_2$ 0.18 mM; trace element solution 5 ml ($ZnSO_4$ x $7H_20$ 3.1 mM, $MnSO_4$ x $1H_20$ 4.5 mM, Fe(HD' citrate 3.0 mM, $CuSO_4$ x $5H_2O$ 0-8 mM); vitamine-nucleotide solution 5 ml (adenosine 6.0 mM, thiamine-HCl 0.059 mM). Sucrose and vitamin-nucleotide solution were sterilized for 45 min at 100°C, the other components were autoclaved (15 min; 121°C).

For data on production of fruit bodies (culture conditions), harvesting mycelia, preparation of mycelial extracts and culture filtrates and for determination of laccase activity see Bresinsky *et al.* (1977).

Disc electrophoresis

pH 4.3 in 7.5% polyacrylamide gel (Reisfeld *et al.* 1962, according to Maurer 1971); pH 8.9 in 10% polyacrylarnid' gel according to Steward and Barber (1964). Before staining* the cylindrical gels were incubated in 0.02 M ammonium acetate for 20 min. Staining for laccase with benzidine (Prillinger 1976).

Slab gel electrophoresis

DESAGA PAG Screening electrophoresis system HAVANNA; method according to Ornstein and Davies (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 //l, staining f°' enzyme activity as described above.

Isoelectric focusing in polyacrylamide gel (Wrigley 1968)

Components of the gel: acrylamide 7.5%, methylene-bis-acrylamide 0.2%, ampholine LKB pH 3-10 or pH 3.5-5.0; chemical polymerization; sample dissolved in 12.5% sucrose and 1% (v/v) ampholine; protecting layer 5% sucrose, 1^ ampholine; electrode fluids: H_2SO_4 1%, NaOH 1%; current: 2 mA/sample, max 400 V; separation time: 3 h;

Figure 1. *Morphology and pigmentation of fruit bodies of the two geographical races* 1s (BRD) *and* 1w (Japan) *and a hybrid* ($1s_4 \times 1w_8$) *of two monokaryotic isolates* ($1s_4$, $1w_8$) *of Pleurotus ostreatus.* For fruit body production and further characterization see Materials and Methods and text.

laccase assay with benzidine as described for disc electrophoresis. For determination of the isoelectric points (IEP) the gel columns were cut in slices of 2 mm length; pH and laccase activity were determined after addition of 1 ml H_2O dist. and homogenization. All enzymatic procedures except electrophoreses were performed at 0-4°C.

Results

1. *Morphological characterization of strains*

(a) ***Fruiting bodies.*** Pleurotus ostreatus has been shown to have a tetrapolar mating system with multiple factors in both the A and B series (Eugenio and Anderson 1968). A statistical evaluation by the same authors leads to 63 different A and 190 different B factors in the whole Population. Monokaryons of the two investigated geographical races Is (BRD) and lw (Japan) used in our experiments did not show any limitation in their outbreeding ability. All matings yielded 100% fertility, indicating different A and B factors. Dikaryotic strains of P. ostreatus fruit well after about 6 weeks using the cultural conditions given by Hilber (1977a) and Bresinksy ***et al.*** (1977). Both the wild strains Is and lw exhibit distinct differences in fruit body morphology (Figure 1), which have been described in detail by Hilber (1977b). Fruit bodies of Is appear singly or in small groups and are characterized by a short and round-bulbous stipe, an irregular, wavy pileus and a dark-brown to black pigmentation, sometimes mixed with a bluish tinge Specially in younger fruit bodies. The margin of the pileus is rolled in. Fruit bodies of strain lw exhibit a tufted growth Pattern, conchoidal to kidney-shaped pilei with a margin which is curved but not rolled in and with an ochraceus to ochre-brownish pigmentation. Fruit bodies of hybrid crosses display an intermediate gene expression as shown by the hybrid $1s_4 \times 1w_8$. Tufted growth pattern, pigmentation and the shape of the pilei are similar to strain lw, a short stipe and a rolled-in margin of the pilei is typical for strain Is.

(b) ***Mycelia.*** A characterization of mycelial growth is given in Tables 1 and 2 and Figure 2. The following observations are made:

1. Using a glucose-peptone agar medium, strain Is is characterized by a loose, well-developed white aerial mycelium. The lw strain differs in showing only a sparsely developed aerial mycelium and a slightly rhythmic growth pattern. The mycelium of the dikaryotic wild strains, therefore, could be easily distinguished from each other (Figure 2).

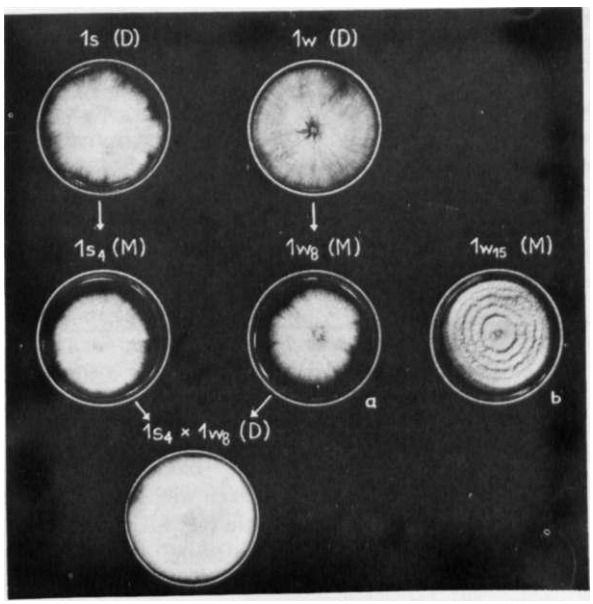


Figure 2. *Growth pattern of Pleurotus ostreatus strains on glucose peptone agar.* Dikaryotic (D) parent strains Is, lw. Monokaryotic (M) isolates $1s_4$, $1w_8$, $1w_{15}$. Dikaryotic hybrid (D) $1s_4 \times 1w_8$. The cultivation time of all strains except of $1w_{15}$ is the same. Different degrees of rhythmicity of growth are shown for two lw monokaryons (a, b). 27°C; continuous light; 7 days. Compare with Table

Table 1. *Growth and laccase activity of the geographical races Is and lw, their monokaryotic progeny, and some hybrid strains of Pleurotus ostreatus.*

Rhythmicity: Presence and amount of rhythmic growth: - absent, + weak (Figure 2, lw, lw_8), ++ medium, + + + strong (Figure * lw_{13}).

For both the geographical races Is and lw the linear growth rate drops after prolonged vegetative propagation in the laboratory (Is from 0.69 to 0.39, lw from 0.67 to 0.47 within a year). In contrast, linear growth rate is stabile in the dikaryotic hybrid strain produced in the laboratory.

Where not stated otherwise, all SE in this table were calculated from at least three determinations. The monokaryons ls_4 and » are not included in the averages, because they have been propagated vegetatively for 1 year longer than the other strains.

		Glucose-peptone agar			Glucose-peptone liquid medium			
			Growth of aerial mycelium[a]		Laccases, U/g d.w.[b]			
Strains (1)	Nuclear phase (2)	Lin. growth rate, cm/day ± SE (3)	Type (4)	Rhythmicity (5)	Extracell. (6)	Intracell. (7)	Dry weight mg/flask (8)	Final pH (9)
Geographical races								
Is (BRD)	D	0.39 ± 0.04	loose, partially interwoven	–	553	225	243	5.3
lw (Japan)	D	0.47 ± 0.03	sparse, partially interwoven	+	746	9	217	5.7
Monokaryotic strains from Is basidiospores								
ls_4	M	0.33 ± 0.02	loose	–	189	102	326	4.1
ls_{17}	M	0.41 + 0.01	loose	+	128	15	150	5.0
ls,«	M	0.35 ±0.01	dense, partially interwoven	–	319	2	298	5.9
ls_{22}	M	0.40 ± 0.03	appressed, interwoven	–	186	19	149	4.6
ls_{23}	M	0.25 ± 0.02	loose, interwoven	–	448	5	184	5.9
ls_{25}	M	0.37 ±0.01	dense	+	60	34	459	3.6
Average:	(*n* = 19)	0.31 ±0.13		6)	222 ± 140	30 ± 37	261 ± 123	4.9 ± 0.9
Monokaryotic strains from lw basidiospores								
lw_8	M	0.33 ± 0.03	dense, interwoven	+	189	12	363	3.8
lw_9	M	0.44 + 0.04	dense, interwoven	+ +	184	51	613	3.7
lw_{10}	M	0.42 ± 0.02	dense, interwoven	+ + +	235	69	389	3.6
$1W_{32}$	M	0.46 + 0.01	dense, interwoven	+ + +	121	8	435	3.9
lw_{15}	M	0.43 + 0.05	dense, interwoven	+ + +	69	6	414	3.9
1"J6	M	0.40 + 0.01	dense, interwoven	+ + +	204	6	439	4.7
Average:	(*n*= 18)	0.42 ± 0.03		(*n* = 6)	167 ±6 1	25 ± 27	442 ± 88	3.9 ± 0.4
Hybrids								
ls_4 x lw_8	D	0.54 ± 0.03	loose, interwoven	–	240	21	323	4.7
ls_{17} x lw_{10}	D	0.47 ± 0.02	loose, partially interwoven	+	279	3	305	5.4
ls_{22} x lw_{10}	D	0.34 ±0.12	appressed	–	169	1	48	5.6
ls_{23} x lw_{10}	D	0.20 ± 0.07	loose, partially interwoven	–	618	4	305	5.9
ls_{23} x lw_{12}	D	0.28 ± 0.02	loose, partially interwoven	+	278	6	216	6.2

2. A comparison of about twenty monokaryons from randomly isolated basidiospores of each wild strain revealed that monokaryons of wild strain Is are very heterogeneous in their growth characteristics. This is quantitatively expressed in a significantly higher standard error of the linear growth rate of Is monokaryons than in monokaryons of the wild strain lw, which exhibit only a small variation. To see the effect of a high degree of heterozygosity on the variation, we crossed monokaryons from Is and lw and evaluated their monokaryotic filial generation for its linear growth rate. Surprisingly, the standard deviation of these monokaryons did not exceed the high value of monokaryotic Is strains, bu was rather intermediate.

3. Especially newly isolated lw monokaryons exhibit * characteristic rhythmical growth pattern (Figure 2, b). This rhythmicity seems to be endogenous, since all monokaryons were grown under constant conditions (continuous Hg"' 27°C).

4. In hybrid dikaryons the growth rate did not always exceed that of their component monokaryons. In contrast to the findings of Wang and Anderson (1972), all the three possibilities, diminished, intermediate, and increased grow

[?]*>le 2. *Growth and laccase activity of the filial generation of the hybrid fruit body* ls$_4$ x lw, *of Pleurotus ostreatus.* For explanation [s]ee Table 1 and the text.

	Glucose-peptone agar			Glucose-peptone liquid medium			
		Growth of aerial mycelium		Laccases, U/g d.w.			
Strain of F,	Lin. growth rate cm/day ± SE	Type	Rhythmicity	Extracell.	Intracell.	Dry weight mg/flask	Final pH
a	0.39 ± 0.02	loose		33	1.5	240	6.04
b	0.30 ±0.01	loose, partially interwoven		61	124	317	4.87
c	0.38 ±0.01	flat, interwoven		156	672	282	5.91
d	0.44 ±0.01	loose, partially interwoven		23	16	513	4.84
e	0.33 ± 0.02	dense, interwoven		39	4.5	300	4.19
f	0.11 ± 0.02	loose		40	1.6	297	5.05
g	0.22 ± 0.02	loose		58	1.7	140	6.05
h	0.27 ± 0.05	loose, interwoven		20	8.8	516	3.90
Average:(A7= 18)	0.33 ± 0.09						

rate were found. Morphologically, hybrid dikaryons resembled the monokaryons of wild strain Is more than lw.

Bresinsky *et al.* (1977), in their studies of the monokaryons ls$_4$ and lw$_8$ found, along with some differences in [t]he overall laccase activity, distinct differences in the respective laccase spectra. After having found certain differences in morphology and growth, we therefore wanted [to] see whether these laccases exhibit genetic differentiation in [tw]o different populations or whether qualitatively altered [z]ymograms may be caused by genetic variability only.

[2]- *Qualitative and quantitative determination of laccase activity*

(a) *Substrate and culture age.* In a first series of experi[me]nts on malt broth medium we determined mycelial growth (dry weight), laccase activity and electrophoretic laccase [s]Pectra over a period of 6 weeks. Samples of the mono[kar]yons ls$_4$, lw$_8$ and the hybrid dikaryon ls$_4$ x lw$_8$ were [an]alysed at 7-day intervals. The data revealed a strong Urease in the extracellular laccase activity within the first *[e]* (the maximum was reached after about 7 days), Allowed by a decrease especially in the dikaryotic strain, [wh]ereas in the monokaryons the laccase activity showed an oscillating behaviour. The intracellular laccase activity did [no]t show significant differences during the time of investigation. Mycelial growth reached its maximum after about 14 days.

[U]sing this malt broth medium, however, difficulties [a]ppeared in the reproducibility of electrophoretic laccase [s]Pectr,. A similar effect was observed by Blaich (pers. [co]mmun.). To overcome this problem we also tried a semi-synthetic glucose-peptone medium and a synthetic sucrose-asparagine medium. The results can be summarized as follo[ws]:

All strains showed the highest extracellular laccase activity (CF) on the malt broth medium. The intracellular laccase activity (ME) from this medium was comparable to that of the glucose-peptone medium.

2. No significant differences in the mycelial growth were observed between the malt broth and glucose-peptone medium. The growth on the synthetic medium, however, was drastically reduced.

3. A comparison of the electrophoretic laccase patterns of independently prepared samples revealed characteristic spectra of excellent reproducibility for all strains grown on the glucose-peptone medium. Contrariwise, the spectra from malt broth medium sometimes contained additional laccase bands. Laccase spectra from strains grown on the synthetic medium differ only quantitatively, showing less activity than those from the glucose-peptone medium. We, therefore, decided to use the glucose-peptone medium for further investigations.

(b) *Growth and enzyme production.* Quantitative extra- and intracellular laccase activity, the mycelial dry weight and the pH in the culture medium of the dikaryotic geographical races Is and lw, their monokaryotic progeny and of dikaryotic hybrid crossings are given in Table 1.

From these data it follows:

1. There are no characteristic quantitative differences, except for a very low intracellular laccase activity (Table 1, col. 7) in the lw strain.

2. In both the dikaryotic wild strains, the amount of extracellular laccase activity (Table 1, col. 6) exceeds the activity found in any one of the monokaryotic offspring. Intracellular^, only the wild strain Is shows a laccase activity significantly higher than in all the other strains (Table 1, col. 7). All the monokaryotic progeny generally exhibits a high variability in intra- and extracellular laccase activity as indicated by a high value for the standard error (Table 1, average cols. 6 and 7).

3. Higher morphological variability was found for the monokaryotic progeny of Is compared to lw (Table 1, cols. 3, 4 and 5). Corresponding findings were made for physio-

logical characteristics. This is quantitatively expressed in a higher standard error in the averages, especially of columns 6 and **9** of Table **I.**

4. Although mycelial dry weight and the acidification of the culture medium (Table **1,** cols. **8** and **9)** are significantly different in the monokaryons from Is and lw, there are no significant differences in the averages of the extra- and intracellular laccase activity.

5. Hybrid crossings between compatible monokaryons reveal that it is possible to improve laccase activity in dikaryons by breeding (compare monokaryons ls_{23} and lw_{11} and their hybrid ls_{23} **x** $lw_{,,}$).

Genetic effects may be responsible for the enhanced laccase activity in dikaryotic strains (hybrid of high-yielding monokaryons) as well as certain physiological differences between mono- and dikaryons. This is supported by the observation that the pH of the medium generally drops less in dikaryotic hybrid cultures than in those of their component monokaryons (Table **1,** col. **9).**

(c) ***Evidence for genetic variation and specific secretion.*** In order to determine whether the observed variability is caused by the presence of different multiple forms of laccase or by different amounts of the laccases produced we used a number of electrophoretic methods.

In preliminary experiments (Bresinsky ***et al.*** **1977),** the main laccase band moved with the front (disc-electrophoresis pH **8.9; 7.5%** gel, R_f**= 1.0).** To position this band well within the separation zone we now used a **10%** gel with a smaller pore size resulting in Rvalues between **0.60** and **0.65.** A comparison of the enzyme spectra of different monokaryons of strains Is and lw after disc electrophoresis at pH **8.9** is given in Figure **3.** There are qualitative differences between both populations, especially for a group of intracellular laccases in the /^-region between **0.1-0.2.** In some spectra there is a higher similarity between monokaryons of different geographical races than between those within the same monokaryotic population (compare monokaryons ls_{23} with $lw_{,6}$ and $lw_{,,}$ with $lw_{,6}$). At pH **8.9** the main laccase band of the culture filtrate of **ls**-derived monokaryons had a somewhat higher **Rvalue** of around 0.65, whereas the main band of the **lw**-derived monokaryons had a somewhat lower **Rvalue** of around 0.60.

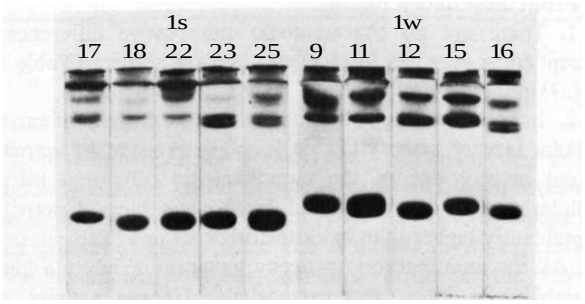


Figure 3. *Evidence for genetic variability in the laccase pattern of monokaryotic populations of the geographical races* Is *and* lw *of Pleurotus ostreatus.* Disc electroporesis pH 8.9, 10% acrylamide; anode at the bottom; all laccase-spectra stained with benzidine for laccase activity.

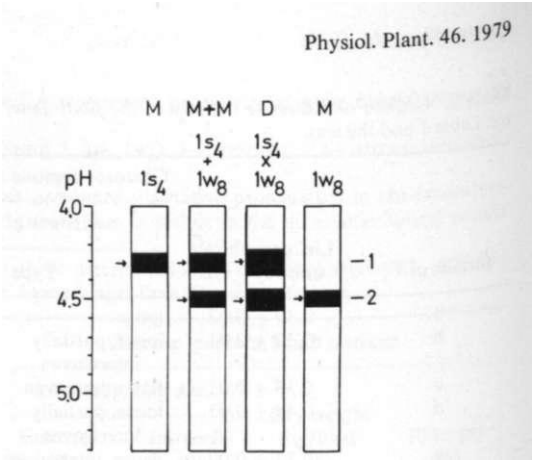


Figure 4. *Isoelectric points of the main laccases in the culture filtrate of the monokaryons* ls_4 *and* lw_8 *in comparison with an vitro' mixture* (ls_4 + lw_8) *and an 'in vivo' hybrid* (ls_4 x lw_8) *of Pleurotus ostreatus.* The figure represents part of an isoelectric focusing pH 3.5-10. The arrows indicate the mean value of the IEP from 5 determinations, the band width gives the standard error of the IEP. For further explanation see Materials and Methods.

In contrast to the very distinct separation of the laccases in the culture filtrate by isolectric focusing, **some** data from mycelial extracts indicate that both enzymes, band 1 and 2< are present intracellular^ as well. There is, however, ^ specific secretion of the enzymes into the culture medium For further investigation, two questions seemed important to us:

1. Are the different laccases in the culture medium of I' and **lw** monokaryons products of a different structural g^^^ or the result of epigenetic differences?

2. Is there any further evidence for a specific secretion **of** laccases into the culture medium?

To exclude the possibility of artifacts by isoelectric focusing such as described by Illingworth (1972), we used in addition slab gel electrophoresis, which gave a better resolution in the range of interest. The results may ^ summarized as follows (Figures 5, 6 and 7):

1. The two laccases detected by isoelectric focusing (bands 1 and 2) are not controlled by the same structural gene in different allelic configurations. In the two g^^' graphical races both enzymes are found in the mycelu*' extract of monokaryons derived from basidiospores with a single nucleus. In the monokaryons from **lw** only lw_8 shows

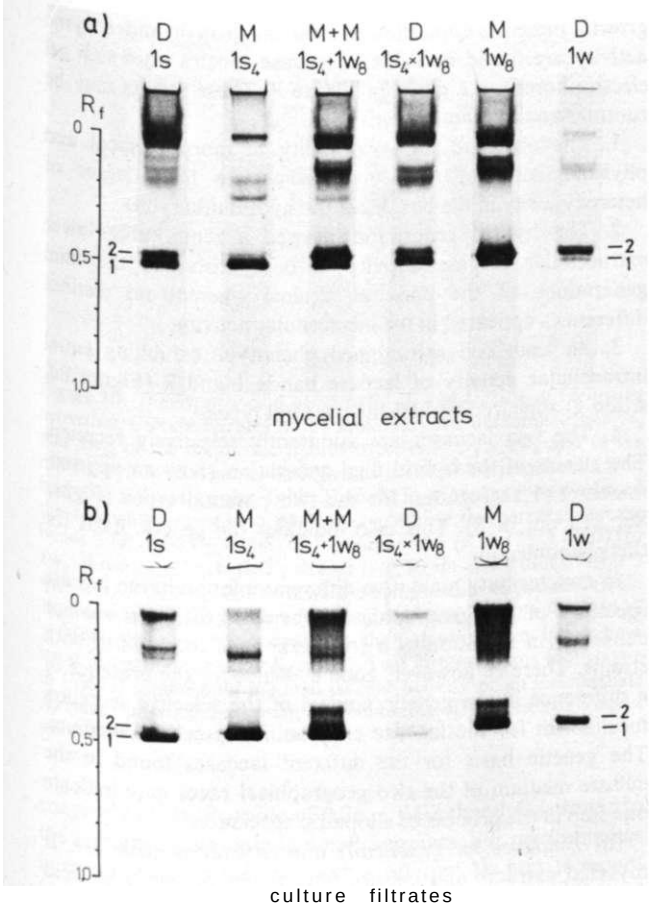


Figure 5. *Laccase spectra of Pleurotus ostreatus after slab gel electrophoresis pH 9.0 of mycelial extracts* (a) *and of culture fixates* (b) *of the geographical races* Is *and* lw, *the monokaryotic Progenies* $1s_4$ *and* $1w_8$ *and of their dikaryotic hybrid* $1s_4 \times 1w_8$. Anode at the bottom. For explanation see text.

both **enzymes** (Figure 5a), whereas in the other ones (Figure 6a) the laccase with IEP of 4.49 (band 2) dominates and the laccase-zyrne with IEP 4.30 (band 1) is detected only after further concentration.

In contrast, all monokaryons of Is (Figure 6a) exhibit at least some activity of both enzymes. Although there was **a** Predominance of strains in which laccase band 1 had the higher activity, strains were also found where both enzymes showed equal activity (Figure 6a, **$1s_{18}$**) or the laccase band 2 Predominated (Figure 6a, **$1s_{17}$**).

2. Comparing the spectra of mycelial extracts with those of the culture filtrates it could be shown for the wild strains (Figure 5b) and then confirmed for the monokaryons (Figure 6b) **that** only one of the two intracellular^ produced laccases is secreted into **the** culture medium. All Is strains secrete **the Coenzyme** band 1, **the** lw strains in contrast secrete **the** enzyme band 2.

3- The result of crossing strains with different secretion

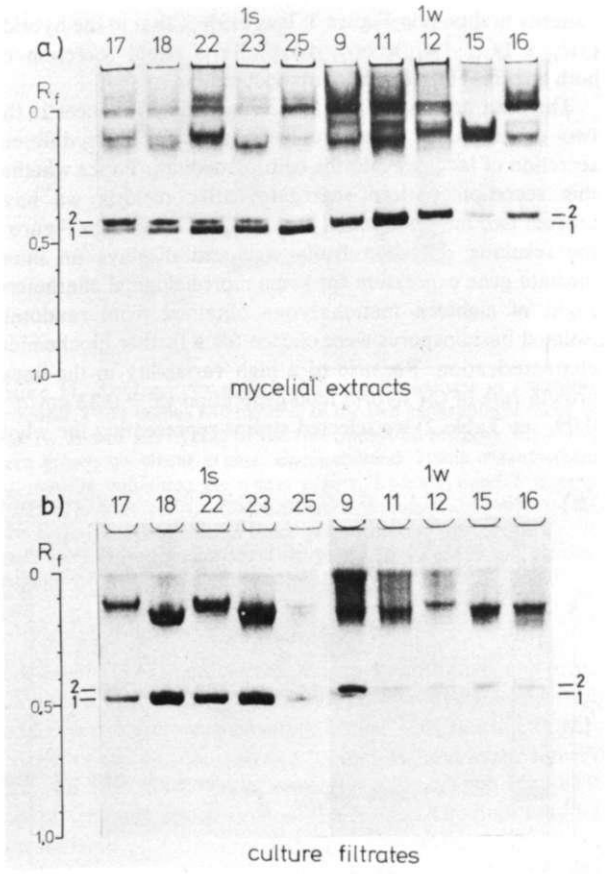


Figure 6. *Evidence that a specific secretion of laccases into the culture medium is different in the two monokaryotic populations of the geographical races* Is *and* lw *of Pleurotus ostreatus.* Slab gel electrophoresis pH 9.0 of mycelial extracts (a) and culture filtrates (b). Anode at the bottom.

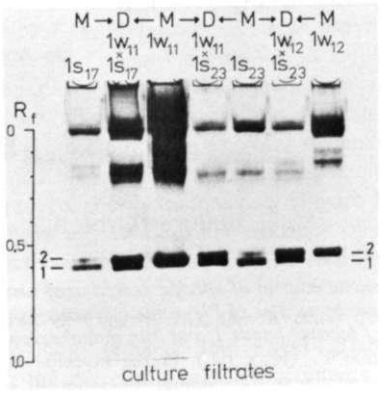


Figure 7. *Evidence for a balanced secretion of laccases into the culture medium by hybrid strains of Pleurotis ostreatus.* In contrast to all monokaryons where the laccase activity in the *R*,0.5-0.6 area is mainly restricted to one band ($1s_{17}$, $1s_{23}$; lw_{11}, lw_{12}), their hybrid dikaryons ($lw_{11} \times ls_{17}$; $lw_{11} \times ls_{23}$; $lw_{12} \times ls_{23}$) exhibit equal laccase activity in bands 1 and 2. Slab gel electrophoresis pH 9.0; anode at the bottom.

patterns is shown in Figure 7. It is evident that in the hybrids ($1w_{8}$ **x** $1s_{17}$, $1w_{8}$ **x** $1s_{23}$, $1w_{12}$ **x** $1s_{23}$) equal secretion of both enzymes into the medium occurs.

The data presented indicate that genetic differences in the two geographical races are responsible for their different secretion of laccases into the culture medium. To see whether this secretion pattern segregates after meiosis we have crossed two monokaryons ($1s_{4}$ **x** lwg). As shown in Figure 1 the resulting dikaryon fruits well and displays an intermediate gene expression for some morphological characters. Eight of eighteen monokaryons obtained from randomly isolated basidiospores were chosen for a further biochemical characterization. Because of a high variability in the linear growth rate of the hybrid filial generation ***(x*** = 0.33 ***cm/d*** ± 0.09, see Table 2) we selected strains representing the whole growth interval. Quantitative data on growth and enzyme activity are found in Table 2, laccase spectra after slab gel electrophoresis are given in Figure 8. These results may be summarized as follows:

1. The observed high variability in morphological and physiological properties may indicate a high degree of heterozygosity in the basidia of the hybrid dikaryon.

2. The hybrid generation showed a remarkably lower extracellular laccase activity in comparison to the fiti^ generations of the parental strains, whereas no distinct differences appeared in the intracellular activity.

3. At least one recombined phenotype exhibiting equal intracellular activity of laccase bands 1 and 2 (Figure 8a, strain g) appeared besides the parental types.

4. The two laccases are apparently selectively secreted. The strains of the hybrid filial generation show an approximately 1:1 segregation for the two parental types (Figure 8b, 5:3 out of 8). This also indicates the genetic basis for this phenomenon.

In conclusion: Qualitative differences found in the laccase spectrum of two geographical races of P. ostreatus are not caused by a mutation of a structural gene common to both strains. There is, however, good evidence for the presence of a difference in the genetic control of the selective secretion mechanism for the laccase enzyme in the culture medium- The genetic basis for the different laccases found in the culture medium of the two geographical races may indicate one step in the process of allopatric speciation.

(d) ***Evidence for genetically altered enzyme structure.*** If mycelial extracts and culture filtrates are analysed by acid disc electrophoresis at pH 4.3, two characteristic bands appear (Figure 9). The band with the lower **/Rvalue** ± 0.007, band 3) is lacking in the mycelial extract. The

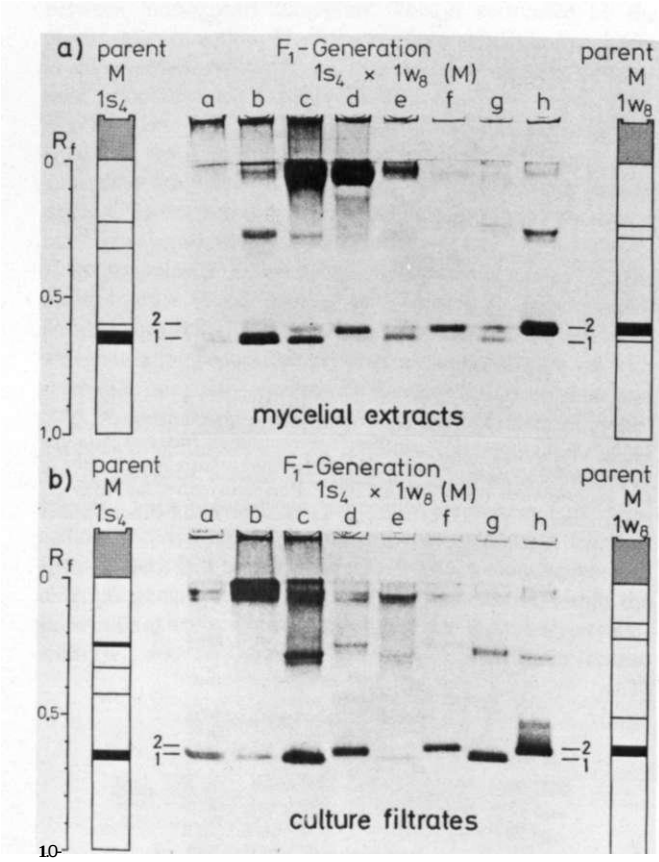
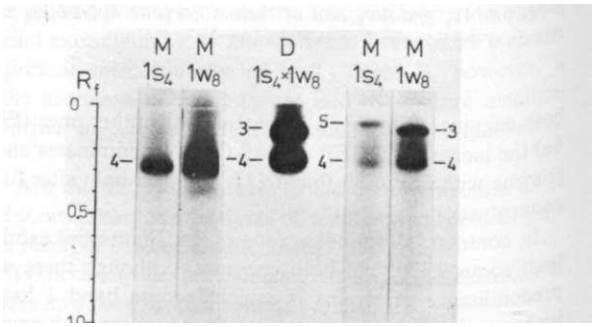
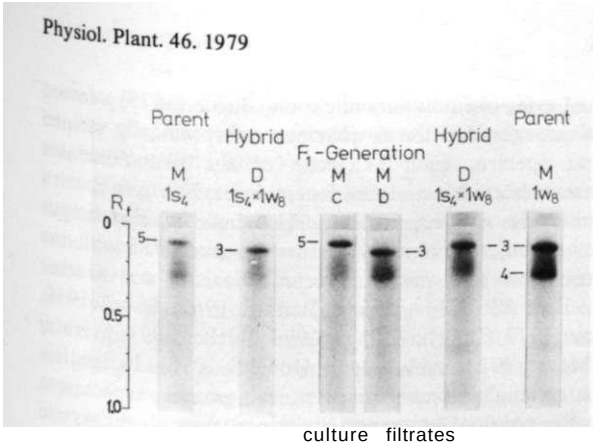


Figure 8. *Genetic control of specific secretion of laccases into the culture medium by strains of Pleurotus ostreatus as evident by the segregation of laccase bands* ***1*** *and 2 in monokaryons* (a-h) *of the filial generation* F, ($1s_{4}$ x $1w_{8}$). In the mycelial extract (a), six strains appear similar to the parental generation (b, c and e to $1s_{4}$; d, f and h to $1w_{8}$). Laccase spectra of two further strains are different showing only one very weak band (a) or an equal activity for both laccases (g). For the secretion into the culture filtrate (b) a 5:3 segregation of these laccases is obvious, although with some quantitative differences. Five strains (a, b, c, e, g) resemble the parent $1s_{4}$, the remaining three strains (d, g, h) are similar to parent $1w_{8}$. Slab gel electrophoresis, pH 9.0; anode at the bottom.

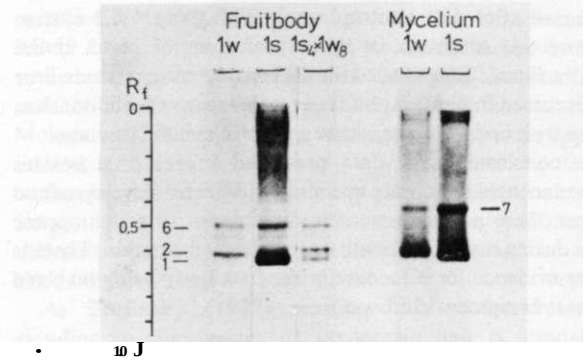


ME FB CF

Figure 9. *Developmental variability of Pleurotus ostreatus* ^ *laccase spectra of mycelial extract* (ME), *fruit body* (FB), *culture filtrate* (CF) *of the monokaryons* $1s_{4}$, $1w_{8}$ *and their dikaryotic hybrid.* Band 3 with the low /Rvalue was characteristic for all culture filtrates of mono- and dikaryons. Only in the mono karyon $1s_{4}$ this laccase is genetically altered (band 5), exhibiting still lower electrophoretic mobility than band 3 (see also Figure l - The laccase band 4 with the /Rvalue around 0.3 was tn predominant laccase of mycelial extracts. In all fruit bodies analyzed, laccase bands 3 and 4 show comparable activities. D electrophoresis, pH 4.3; cathode at the bottom.



culture filtrates



10 J

Figure 10. *Inheritance of a genetically altered laccase of Pleurotus ostreatus.* Culture filtrates of the monokaryotic parental strains ls_4 and lw_8, of the dikaryotic hybrid mycelium $ls_4 \times lw_8$ and of the monokaryotic filial generation (strains b and c), derived from a fruit body $ls_4 \times lw_8$ (see also Table 2). Eight F_1-monokaryons displayed the following segregation pattern concerning the parental laccase bands 5 (R_f 0.085 ± 0.008) of ls_4 and band 3 (R_f 0.125 ± 0.007) of lw_8: three monokaryotic F_1 strains *(e.g.* strain c) contained band 5 a R_f of 0.085 ± 0.008, therefore being similar to parental strain ls_4; three isolates *(e.g.* strain b) contained band 3 with a R_f of 0.117 ±0-009, therefore being similar to the parental strain lw_8 (whose band 3 is within the same grange). Two further strains (not shown £ Figure) exhibit only minimal laccase activity or an intermediate value, possibly indicating recombinants. Disc electrophoresis, pH 4.3; cathode at the bottom.

Figure 11. *Evidence for a fruit body-specific laccase in Pleurotus ostreatus.* Fruit bodies and mycelia of the two geographical races 1 s and lw and of the hybrid of the monokaryotic progeny $ls_4 \times lw_8$ were grown on wheat grains. Laccase band 7 was characteristic for mycelia cultivated on wheat grains. Laccase band 6 is only detected in fruit bodies. Laccase bands 2 and 1 are found in the fruit bodies and in mycelial extracts and culture filtrates of all the other media used (compare Figures 5, 6, 7). Slab gel electrophoresis, pH 9.0; anode at the bottom.

zyme with an **/Rvalue** of 0.26 ± 0.02 (band 4) is found in the culture filtrate only in small amounts, but predominates in the mycelial extract. Of twelve different lw and ls monokaryons analysed, we detected only one strain (ls_4) whose laccase (band 5 with R_f 0.085 ± 0.008) was significantly Afferent from the /^-average of the band 3-type laccases. We confirmed this result by analysing the hybrid filial generation $ls_4 \times lw_8$ as indicated in Figure 10. Although the genetically altered enzyme did not appear in the dikaryotic hybrid, segregation for the two parental types occurred in the monokaryotic progeny. Of eight strains analysed, six exhibited a parental 1:1 segregation pattern.—Of the remaining two strains one shows a band of very low laccase **Activity** whereas the other one shows a laccase with an ***Rf*** value intermediate between the two parental enzymes. This will be investigated further.

(e) ***Evidence for developmental variation of laccase Patterns.*** If phenoloxidases play a role in fungal morphogenesis (Hirsch 1954, Phillips and Leonhard 1976, Prillinger and Esser 1977) one would expect quantitative and even qualitative differences between vegetative mycelia and fruit bodies. We, therefore, measured laccase activities in CF, ME and FB of the two wild strains ls and lw and in the hybrid dikaryon $ls_4 \times lw_8$. Table 3 (enzyme activity) and Figures 9 and 11 (enzyme spectra) show the results. The data may be summarized:

1. In these experiments wheat grains were used as the substrate for fruit body production. The two laccases characterized in the mycelial extract (Figure 5a, bands 1 and 2) were also present in the fruit body. Fruit bodies of wildstrain ls contain the enzyme with an IEP of 4.30 (Figure 11, band 1), whereas fruit bodies of wild strain lw contain the enzyme with an IEP of 4.49 (Figure 11, band 2). Fruit bodies of the dikaryotic hybrid $ls_4 \times lw_8$ contain both enzymes (Figure 11, bands 1 and 2).

2. A comparison between the zymograms of the fruit bodies and the mycelial extracts revealed an additional laccase band (Figure 11, band 6) which occurs only in the fruit bodies. On the other hand, a distinct additional band was found in mycelial preparations only (Figure 11, band 7). This band, however, is a further evidence of an enzyme induced or activated by the substrate as mentioned for the malt broth medium.

In this series of experiments, the mycelial extracts were derived from homogenizing the wheat grain substrate together with the mycelia. Control experiments with wheat grains only gave no laccase activity and spectra at all.

3. A quantitative determination of the laccase activity showed distinctly higher values for the fruit bodies of strain ls than for lw (Table 3). This correlates well with the darker pigmentation of strain ls compared to lw (Figure 1).

4. Characteristic differences in the laccase activities

Table 3. *Laccase activity in the fruit bodies of the geographical races* ls am/1 w *and hybrid* $ls_4 \times lw_8$ *of Pleurotus ostreatus.*

Strains	Laccase U/g d.w.	Fruit body % dry weight
ls	6.0	11.1
lw	1.0	14.9
$ls_4 \times lw.$	2.1	23.5

appeared after disc electrophoresis with the pH 4.3 system (Figure 9). Although in the mycelial extract and in the culture filtrate one of the two different laccases (bands 3 or 4) discussed in paragraph (d) of results always predominates, in the fruit body both enzymes appear in similar amounts.

In conclusion: The data presented suggest that besides some laccases which are specific for different developmental stages, there are characteristic multiple forms which appear only during mycelial growth or fruit body formation. There is some evidence for a laccase in the fruit body being involved at least in pigmentation.

Discussion

To investigate variability within different organisms one has to consider three kinds of individual variation: (1) Variations which commonly occur during different developmental stages. (2) Variations which may depend on the environment *(e.g.* induction *etc.).* (3) Variations which are caused by differences in the genetic material. Especially in fungi, one further aspect should not be overlooked. It arises from the question: Does the mycelium of a fungus represent an individual, in the same sense as plants grown from seeds, or does it represent a population of frequently genetically different nuclei in hyphae?

Evidence for the latter aspect is given by the Buller phenomenon (Buller 1931, Quintanilha 1937) and by some experimental observations in the monokaryotic progeny of *Agrocybe aegerita* (Esser *et al.* 1974).

A comparison of morphological and growth characteristics, especially the linear growth rate, using approximately twenty randomly isolated monokaryons from the two wild strains indicated a significantly higher genetic variation in the progeny of Is, whereas monokaryons of the lw strain are more homogeneous as indicated by a smaller standard error.

If the variation within the monokaryotic progeny of a fruiting dikaryon is only caused by the degree of heterozygosity of the meiotic nuclei in the basidia, one would expect the highest variation in the progeny of a hybrid cross of Is x lw. As evident from Tables 1 and 2 the standard deviation of the hybrid progeny exhibits a variation only within the values of Is and lw. A simple explanation for this observation could be that hyphae of the Is wild strain especially represent a dikaryon, which is composed of a population of cell-like compartments frequently having different nuclei (see above). Further work is now in progress to see whether it is possible to detect a heterokaryotic origin for a wild strain by means of this statistical method.

A heterokaryotic origin of both wild strains becomes more probable with the observation that the linear growth rate drops after prolonged vegetative propagation in the laboratory; in contrast, it does not drop in the $ls_4 \times lw_8$ hybrid dikaryon.

Addressing the problem of demonstrating variation in fungi, Bresinsky (1977) discussed some limitations in the value of using chemotaxonomic tools. Blaich (1977) stressed the advantages that the employment of specifically stained enzyme spectra, such as those of the phenoloxidases (laccases), esterases, and aminopeptidases, has over spectra of uniformly stained proteins. The bulk of the fungus enzyme-chemotaxonomic literature is concerned with the phyco-, asco- and deuteromycetes (Nealson and Garber 1967, Hall 1967, Meyer and Renard 1969, Stibes 1970, Nasuno 1972, Stout and Shaw 1973, Bucher 1974, Selvaraj and Meyer 1974, Abbott and Holland 1975). In the few works on higher basidiomycetes, the genetic aspect was generally overlooked, especially since mostly dikaryotic strains only were compared (Lawson *et al.* 1975; Blaich and Esser 1975; Blaich 1977). Since it is known that monokaryons of the same parental strain may exhibit different morphological phenotypes (Esser *et al.* 1974, Stahl and Esser 1976, Williams *et al.* 1976, Esser and Meinhardt 1977) one could expect genetic variation for different enzymes, too. The two allopatric wild strains of Pleurotus ostreatus studied had different natural substrates; Is was isolated from dead trees in Germany *(Fagus sylvatica),* lw is an industrial strain, whose natural substrate must be different from that of Is (Mori, pers. comm.).

To evaluate the suitability of laccase spectra for chemotaxonomic work, one of the main questions was whether all of the multiple enzyme bands of the spectrum of a singj° species are equally influenced by genetic and epigenetic factors and, therefore, are all equally suited for chemotaxonomic purposes, as is commonly assumed and practised. It has been shown that in fungi the multiple laccase bands in electrophoreses may arise from several individual laccases, which differ in quaternary structure, molecular weight amino acid composition and carbohydrate content (Molitofl[5] 1976, 1978) and which in addition are genetically controlled (Froehner and Eriksson 1974a,b, Prillinger and Esser 1977). If, however, individual enzyme bands react individually [00] these factors, this would require a much more detailed knowledge of the intraracial and intraspecies variability of such spectra in order to make chemotaxonomically relevant statements. Considering our results we can draw the following conclusions:

1. Using different kinds of electrophoretic methods we were able to detect at least nine multiple laccases in the fungus Pleurotus ostreatus. This is in good agreement with data obtained from analytical isoelectric focusing by Blaich and Esser (1975).

2. A group with approximately three laccase bands with low /^-values in the basic separation gel (Figures 3, 5 and 6) demonstrates the presence of a high degree of genetic variation among monokaryotic isolates of both wild strains. To this group also belong the two laccase bands which, according to Bresinsky *et al.* (1977), are different for the monokaryons ls_4 and lw_8.

3. One further laccase band (Figure 11, band 6) appe[arr(]* only in fully developed fruiting bodies thus showing a high

correlation with development. In addition, two other laccases display quantitative variation in different developmental stages (Figure 9, bands 3 and 4).

Using different types of media, we have detected two further laccase bands highly dependent upon environmental conditions. One is shown in Figure 11 (band 7), obtained from strains cultivated on wheat grains, the other one was generally found in strains grown on malt broth medium. Although used by a number of authors for reasons of simplicity and good growth, and although phenoloxidase Production was stimulated considerably, we discontinued the use of malt broth medium because the electrophoretic spectra showed poor reproducibility. One of the reasons might be uncontrollable changes of the medium during autoclaving, often resulting in dark pigments. Inducibility of laccase *(e.g.* by phenolic compounds) was shown by Froehner and Eriksson (1974a), Leonowicz and Tro-Janowsky (1975a,b) and others.

Of the observed multitude of laccase bands in the two P. ostreatus strains only a few seem to be useful for chemotaxonomic studies: (a) The laccase bands 1 and 2 with Rvalues between 0.4 and 0.5 in the basic separation gel figures 5 and 6) and (b) two further bands from the acid disc electrophoresis whose quantity is correlated with developmental stages (Figures 9, bands 3 and 4).

Except for the monokaryon ls1, where a probably mutatively altered laccase is found in the acid separation gel figures 9 and 10, band 5), no genetic variation within ls or lw was observed in the bands 1, 2, 3 and 4. Preliminary results from other P. ostreatus strains and other Pleurotus species suggest that the /^locations of the above-mentioned laccase bands 1 and 2 may also be important chemotaxonomically, in one case showing identity or a closer relationship *(e.g.* with *P. columbinus),* in the other case Seating distinct differences *(e.g.* against *P. cornucopiae, P. cystyngii* and *P. pulmonarius).*

Considering the genetic aspect of our results, we were able to demonstrate that selective secretion of an enzyme into the culture medium may phenocopy qualitative alteration in the enzyme structure in monokaryons of Is and lw strains figure 6). Although genes are known which somehow regulate the synthesis of phenoloxidases (Horowitz *et al.* 1961, 1970a,b, Fox *et al.* 1963, Froehner and Eriksson 1974a,b, Hanson and Brody 1975, Ander and Eriksson 1976) or have some influence on laccase structure (Prillinger 1976, Prillinger and Esser 1977), nothing has previously keen known about the specific secretion of these enzymes, ^e similarity or even identity of intra- and extracellular ^ceases, however, indicates that the enzyme is first Produced intracellularly and then secreted without alteration (Froehner and Eriksson 1974b, Molitoris 1976).

As indicated quantitatively in Table 1 and shown by electrophoretic experiments (Figure 7), we are able to increase laccase production by crossing selected high-yielding monokaryotic strains and can overcome selective secretion by using hybrid strains secreting both laccases, bands 1 and 2, in equal amounts. Because it is known from white rot fungi that extracellular laccases participate in lignin and cellulose degradation (Ander and Eriksson 1976, Molitoris 1978), our breeding results may be of some applied interest.

Besides this apparent regulatory phenomenon, one monokaryon with a structurally altered laccase exhibiting a different /Rvalue after disc electrophoresis was also identified (Figures 9 and 10, band 5).

As Bresinsky (1977) mentioned, a profound understanding of the species as taxonomic unit is impossible without information about the evolutionary processes which result in speciation.

Kemp (1975, 1977) working with *Coprinus,* and Jurand (1975, as cited in Kemp 1977) working with *Psathyrella* suggest that sympatric speciation at the cellular level may be common in basidiomycetes and that they may differ in this respect from angiosperms and animals, where external or allopatric speciation occurs. Kemp was able to detect a genetic isolating mechanism similar or identical to heterogenic incompatibility (for references see Esser and Blaich 1973) in basidiomycetes and considers this process to be the first step in speciation. According to Kemp, morphological, ecological and other differentiation processes take place after mating has been genetically blocked. In contrast we found that two geographical and ecological races of P. ostreatus (ls, lw) are still compatible, although a number of differences have developed. Morphological divergencies are found in the mycelial growth pattern (Figure 2, Table 1) and in the fruit bodies (Figure 1). Physiological differences concern the growth rate (Table 1) and the production of two extracellular laccases (band 1 and 2), which are controlled by a specific secretory mechanism showing Mendelian segregation.

Each of the two wild strains ls and lw and their monokaryons, derived from uninucleate basidiospores, produces both of these laccases intracellularly. The two geographical races can be distinguished, however, by their secretion of either one or the other form of the enzyme (Figures 5, 6 and 8). At this point one is tempted to speculate in terms of a common ancestor for both laccases which have followed divergent evolution after gene duplication. It is possible that accumulated point mutations and a different substrate determined which enzyme was better adapted; thereupon a specific secretory mechanism may have developed (Dobzhansky *et al.* 1977). Further insight, however, will be obtained only after some kinetic data on the two extracellular laccases are available.

Our morphological and biochemical data, however, indicate that not only sympatric but also allopatric speciation can occur in higher basidiomycetes, although it may often have been overlooked. Further experimentation along this line could contribute to a better understanding of micro-evolutionary processes.

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