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Variability of Laccase Activity in the White-Rot Basidiomycete *Pleurotus ostreatus*

P. BALDRIAN, J. GABRIEL

Laboratory of Biochemistry of Wood-rotting Fungi, Institute of Microbiology, Academy of Sciences of the Czech Republic, 142 20 Prague, Czechia

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ABSTRACT. The production of laccase in liquid cultures of the white-rot fungus *Pleurotus ostreatus* was highly variable. During the first days of cultivation, the relative variability was as high as 80–100 % and it decreased to 30 % in the course of cultivation. The main source of variability was assumed to be the independent development of enzyme activity in individual cultures. Cultures with high laccase production showed also high production of the other ligninolytic enzyme – Mn-dependent peroxidase. The variability was probably due to the source of inoculum, deactivation of the enzyme in culture liquid and genetic variations among the cultures. Variability of laccase activities was lower during solid-state fermentation on wheat straw and during the growth in nonsterile soil.

Abbreviations

Avg – average; CCBAS – *Culture Collection of Basidiomycetes* (*Institute of Microbiology, Academy of Sciences of the Czech Republic*, Prague); Lac – laccase; LOM – loss of organic matter; MnP – manganese peroxidase; RSE – relative standard error; SE – standard error.

Laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) is an important component of the lignin-degrading enzyme complex of the white-rot fungi, participating in the degradation of wood components. In the presence of redox mediators, laccase can also oxidize nonphenolic substrates (Bourbonnais and Paice 1990) and thus it is proposed for the use in xenobiotics degradation (*e.g.*, Eggert *et al.* 1996; Novotný *et al.* 1999; Kahraman and Yeşilada 2001; Zilly *et al.* 2002). Therefore, it is useful to understand the regulation of laccase production in fungal cultures.

In the past, much attention has been paid to the factors affecting laccase activity in fungal cultures. Laccase is produced during the secondary metabolic phase of growth (*e.g.*, Hattaka 1994; Leonowicz *et al.* 2001). The gene expression and enzyme production is influenced by culture media composition (Buswell *et al.* 1995; Yaver *et al.* 1996; Ohga and Royse 2001) or by the addition of inducers such as copper (Collins and Dobson 1997; Baldrian and Gabriel 2002).

The estimation of laccase activity in the absence of inductors often reveals high variability among replicates. This represents a serious limitation in the interpretation of experimental data. So far, neither the level of variability of enzyme activity, nor its causes have been studied. The aim of this study was to quantify the variability of laccase production in the white-rot fungus *Pleurotus ostreatus*, a model organism for the degradation of oligocyclic aromatic hydrocarbons (PAH), in liquid cultures. The possible sources of the variability have been addressed.

MATERIALS AND METHODS

Organism and culture conditions. The wood-rotting basidiomycete *Pleurotus ostreatus* strain CCBAS 477 was used. The fungus was maintained on slant GC agar (in g/L: glucose 20, corn-steep 7, agar 25; pH 6). Static cultivations in a liquid mineral medium were performed in 250-mL conical flasks containing 40 mL of nitrogen-limited medium (Tien and Kirk 1988). Flasks were inoculated with two 7-mm agar plugs from the edge of an actively growing colony, precultivated on the same medium for 8 d at 28 °C. The liquid stationnary cultivation proceeded at 28 °C in the dark. The experiment was run in 38 replicates. To study the effect of inoculum, flasks with liquid media were inoculated with two 7-mm agar plugs of mycelia from the edge of an actively growing colony, or with the plugs covered by oldest mycelium, cut from the center of the agar plate. Ten replicates were run for each treatment.

The cultivation of *P. ostreatus* on wheat straw proceeded in 100-mL conical flasks containing 5 g air-dried milled straw (particle size 2–8 mm). The straw was moistened with 15 mL distilled water (final water content 75 %), the flasks were stoppered with cotton plugs, autoclaved (2 × 121 °C for 30 min) and inoculated with two agar plugs with mycelium. The cultures were incubated at 28 °C in the dark. The cultivation in nonsterile soil was done according to Baldrian *et al.* (2000).

Sampling procedure. Each sampling day, 500-μL aliquots of the culture liquid were sterilely withdrawn from liquid cultures, filtered and used for enzyme assays. At the end of cultivation, culture liquid was filtered and the dry mass of mycelia was estimated after drying at 105 °C to constant mass. The straw and soil cultures were collected on different days of incubation, and the enzymes were extracted (Baldrian and Gabriel 2002; Baldrian *et al.* 2000). Five replicates were used for straw and soil cultures.

Enzyme assays. Lac activity was measured by monitoring the oxidation of ABTS (2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid) in citrate–phosphate (100 mmol/L citrate, 200 mmol/L phosphate) buffer (pH 5) (Niku-Paavola *et al.* 1990). Activity of MnP was assayed according to Ngo and Lenhoff (1980) in succinate–lactate buffer (both 100 mmol/L, pH 4.5). All measurements were done in 10-mm quartz cuvettes using a UV-VIS spectrophotometer (Lambda 11, *Perkin-Elmer*) and evaluated using UV-Winlab software from the same manufacturer. One unit of enzyme activity (U) was defined as the amount catalyzing the production of 1 μmol/mL colored product per min.

Statistical evaluation of results. Statistical analyses (linear regression, Student's *t*-test, and the frequency analysis combined with χ^2-test for distribution fitting) were performed by the use of statistical software Microcal™ Origin™ 5.0 Professional (*Microcal Software*). RSE was calculated as RSE = SE/Avg.

RESULTS AND DISCUSSION

During stationary cultivation of *P. ostreatus* on liquid media Lac activity started to rise after 6 d and increased until the end of the experiment (Table I). Highest variability was detected during the first days of growth (80–100 %), whereas it decreased to 35–30 % in the last days of cultivation. The distribution of enzyme activity in different sampling periods fitted the normal distribution (Fig. 1).

Table I. Statistical characteristics of Lac activity in liquid stationary cultures of *Pleurotus ostreatus* (n = 38)

Day	Avg U/L	SE		Median U/L	Minimum U/L	Maximum U/L
		U/L	%			
3	0.15	0.16	105	0.12	0	0.60
6	0.46	0.40	86	0.45	0	1.35
10	4.18	1.59	38	4.04	1.56	8.76
13	10.1	3.70	37	10.4	3.24	18.2
17	16.8	6.40	38	15.4	6.05	36.2
20	19.4	6.54	34	18.3	10.02	37.4
24	18.9	7.10	38	17.7	11.36	41.0
27	27.1	10.5	39	24.9	14.48	62.9
31	40.2	12.1	30	39.5	20.85	69.4

When the activities of samples from the same replicates taken on different days of cultivation were correlated it became obvious that the activity in different flasks develops highly divergently (Fig. 2). The flasks with high Lac activity on one sampling day showed on average a higher activity on subsequent sampling days. This dependence was found to be strongly correlated (Table II). The time course of enzyme activity in a particular flask is already determined early in the development of the culture. The samples were divided into two groups according to their Lac activity on day 31: A – replicates with lowest Lac activity and B – replicates with the highest activity and an average Lac activity were calculated for both groups. The two groups differed already on day 10 ($p < 0.02$; *t*-test value 0.017). On day 13, the difference between the two groups was already significant at 0.1 % probability level (*t*-test 0.0004). Similarly, when samples were divided according to the activity on day 10 into groups C – the samples with lowest Lac activity and D – the samples with the highest Lac activity, the development of Lac activities in the two groups was independent (Table III).

Since the first differences in Lac activity development in individual flasks were already recorded at the very beginning of the fungal culture development, the question was addressed, whether there can be any role of the inoculum on this phenomenon. This was partially confirmed in the experiment where liquid stationary cultures were inoculated with either fresh or older inoculum (7–8 d). After 13–17 d of incubation, Lac activity in flasks inoculated with fresh mycelium was 1.6× higher than that in the flasks with old inocula ($p < 0.05$). Another possible factor contributing to the development of Lac activity is the inactivation of produced enzyme. Activity of Lac present in the culture liquid decreased with time (Baldrian and Gabriel 2002), probably due to the action of proteinases and other extracellular factors (Böckle *et al.* 1999; Palmieri *et al.* 2001).

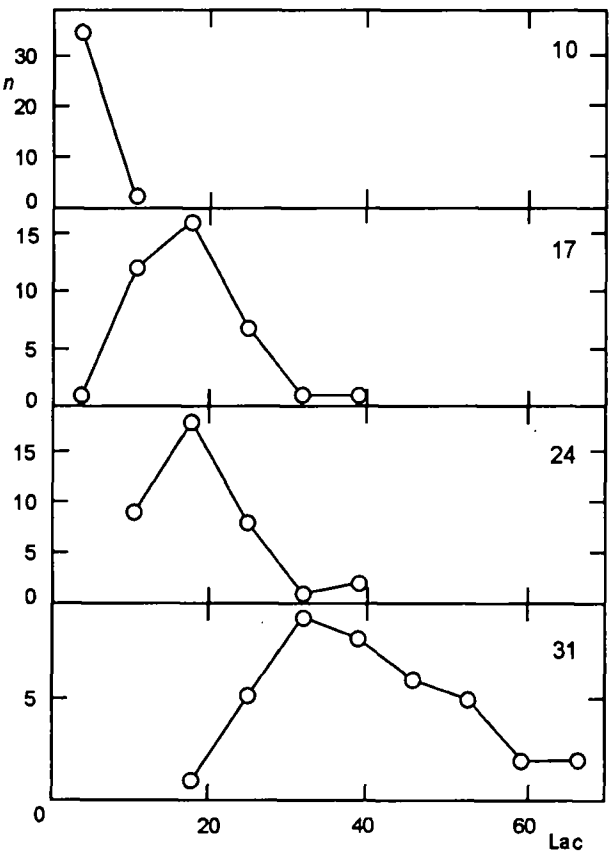

Fig. 1. Distribution of *Pleurotus ostreatus* Lac activity (U/L) in the samples (n) of culture liquid collected on days 10, 17, 24 and 31; $n_{tot} = 38$; for further details *see* text.

Table II. Correlation coefficients of *Pleurotus ostreatus* Lac activity ($n = 38$) on different samplings days (6–31 d), and the probabilities (p) of the hypothesis[a] that variables are independent

Day	6	10	13	17	20	24	27
10	0.587**						
13	0.377*	0.874***					
17	0.204	0.635***	0.870***				
20	0.343*	0.717***	0.873***	0.903***			
24	0.279	0.634***	0.712***	0.736***	0.855***		
27	0.367*	0.643***	0.683***	0.590***	0.804***	0.861***	
31	0.237	0.538**	0.651***	0.657***	0.836***	0.803***	0.846***

[a]* $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$.

The variability of enzyme activity was higher compared to the fungal growth. In liquid culture, where average RSE of Lac activity estimated on the same day was higher than 40 %, the average SE of mycelial dry mass was 16 %. The variability of Lac activity was lower when the fungus was cultivated on

wheat straw – RSE 20 % – and also during the growth in nonsterile soil (also 20 %). However, the measure of fungal growth in the straw substrate – the loss of dry mass – was still less variable under these circumstances (average RSE 3 and 11 %, respectively). During growth in soil, the differences between samples

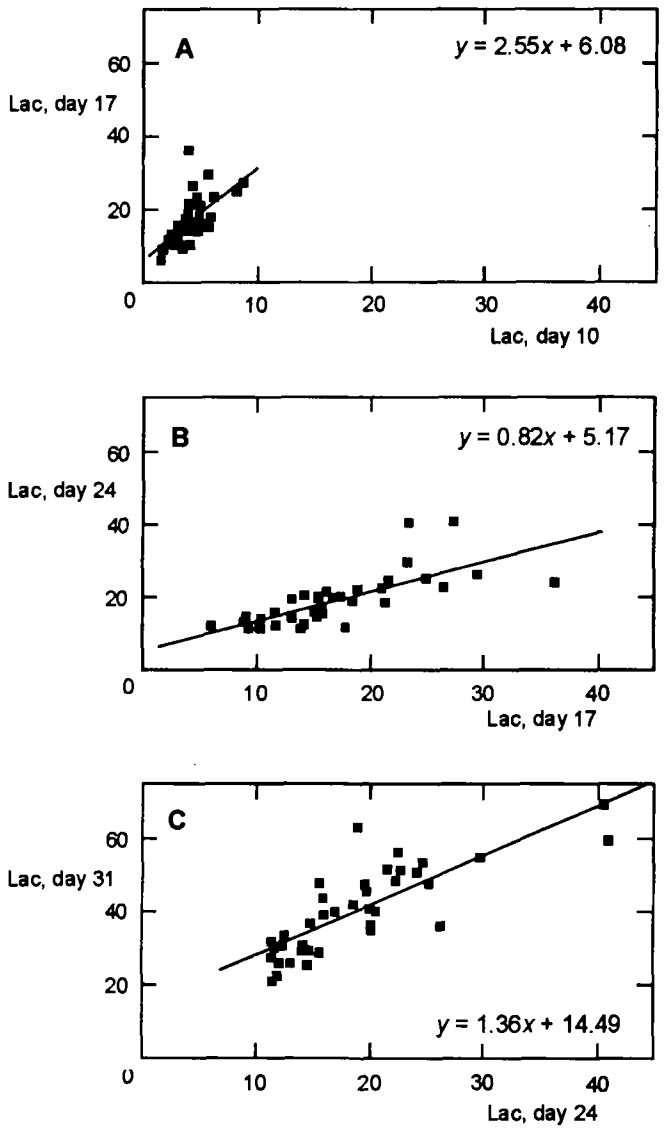

Fig. 2. Plot of *Pleurotus ostreatus* Lac activity (U/L) in the samples of culture liquid; **A** – day 17 to day 10, **B** – day 24 to day 17, **C** – day 31 to day 24; $n_{tot} = 38$.

Table III. The time course of *Pleurotus ostreatus* Lac activity in the groups of replicates A–D[a]

Day	Activity, U/L			
	A	B	C	D
10	3.64 ± 1.40	4.73 ± 1.62	**2.99 ± 0.77**	**5.38 ± 1.27**
13	8.15 ± 3.25	12.0 ± 3.13	7.68 ± 2.77	12.5 ± 2.89
17	13.5 ± 4.99	20.0 ± 6.02	13.9 ± 6.30	19.6 ± 5.25
20	15.3 ± 4.16	23.4 ± 6.02	16.0 ± 5.17	22.7 ± 6.12
24	14.6 ± 3.84	23.1 ± 7.10	16.0 ± 3.90	21.7 ± 8.44
27	19.8 ± 3.26	34.4 ± 10.1	22.4 ± 6.49	31.8 ± 11.7
31	**30.2 ± 5.00**	**50.2 ± 7.99**	34.5 ± 9.44	45.9 ± 11.9

[a]Group A – lowest on day 31, B – highest on day 31, C – lowest on day 10, D – highest on day 10 (the samples were grouped into groups A and B, respectively, C and D on the basis of enzyme activity on the day printed in **bold**); means ± SE; $n = 19$ for each group.

were the highest during the first week of soil colonization (RSE of Lac activity 34 and 22 % in the case of LOM); later, the differences reached a constant value of RSE about 15 % in case of Lac activity and 8 % in case of LOM.

Part of the variability can be ascribed to genetic and physiological differences among cultures. The differences among isolates derived from basidiospores of *P. ostreatus* were substantially higher than in the dikaryotic mycelial colonies of the parental strain, as well as among the protoplast-derived isolates of the same strain (Eichlerová-Voláková and Homolka 1997). In this case, the difference was considered to be caused mainly by the monokaryotic nature of the isolates. Another treatment, affecting genetic information of the fungus – UV mutagenization – has also been shown to increase the variability of enzyme production (Homolka *et al.* 1995). The change of genetic information of fungal cultures, such as dedikaryotization or sectors formation, occurs frequently and it might well account for part of the total variability.

Regression analysis showed that Lac activity is highly positively correlated with the activity of MnP. On day 9, activities of the two enzymes were correlated with $R^2 = 0.532$, and $p < 0.001$. This can be explained as an earlier onset of the secondary metabolic phase, during which both enzymes are produced. On the other hand, the dry mass of mycelium after cultivation (day 31 of the experiment) and Lac activity on the same day were found to be independent ($R^2 < 10^{-4}$); this corresponds to the stationary developmental stage of the fungus.

Since laboratory experiments with ligninolytic enzymes are necessary for the study of the biotechnological use of white-rot fungi, it is reasonable to ask which experimental design (number of replicates) will give reliable results. It is clear that the quality of results is dependent both on the variability of the estimated value and on the number of samples (replicates) taken. With values showing a high level of variability, reliable values can be obtained only when appropriate number of replicates is used. The data of Lac activity in liquid cultures of *P. ostreatus* on day 13 ($n = 38$, Avg = 10.07 U/L) were taken as a representative – though limited – set, showing the distribution of Lac activity at that time point. From this set, subsets containing 1, 2, 3, ... , 30 samples were generated and corresponding averages were calculated. The distribution of these calculated averages is shown in Fig. 3. It represents the quality of estimates of average Lac activity obtained with different number of samples (flasks). It is apparent that, for example, on taking two samples from a set, the error of average estimation will be ±18 % with $p = 0.50$, but with $p < 0.02$ it will be more than ±57 %. If five samples are taken instead of two, the respective errors will be ±10 and ±36 %, and with 10 samples ±7 and ±23 %. Using the data shown in Fig. 3, two principal questions can be answered: (*i*) how many samples are necessary to ensure a certain level of accuracy of the results and (*ii*) how accurate is the result when a specified number of samples was used. It has to be noted that calculations based on other days of estimations should provide slightly different values, depending on the relative variability of the value estimated.

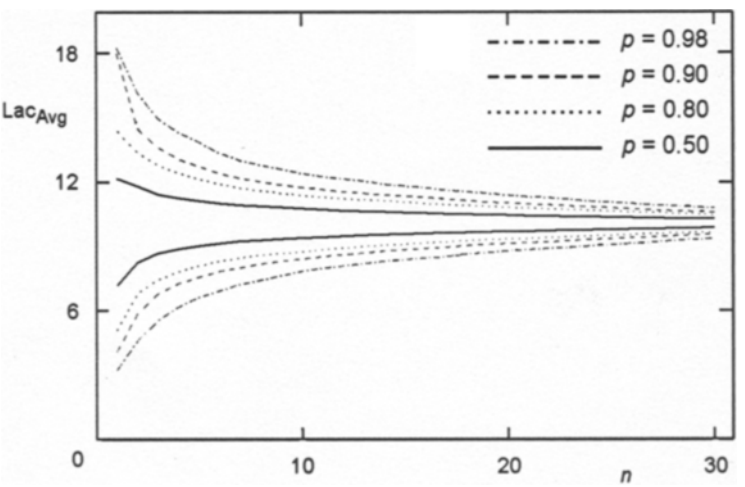

Fig. 3. The accuracy (p) of average Lac activity (Lac_{Avg}, U/L) estimation in dependence on the number of replicates; the lines represent a range within which the estimate lays; calculated from the data on *Pleurotus ostreatus* Lac activity in 13-d-old cultures; $n = 38$; for further details *see* text.

The variability among the estimates of enzyme activity cannot be completely overcome. In fact, it is one of the characteristics of the fermentation. This has to be taken into account when designing an experiment or a technological procedure.

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