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EXPERIMENTAL ARTICLES

Interrelation between the Composition of Lipids and Their Peroxidation Products and the Secretion of Ligninolytic Enzymes during Growth of *Lentinus* (*Panus*) *tigrinus*

D. A. Kadimaliev[a, 1], O. S. Nadezhina[a], N. A. Atykyan[a], V. V. Revin[a], and V. D. Samuilov[b]

[a] *Ogarev Mordovian State University, ul. Bol'shevistskaya 68, Saransk, 430000 Russia*
[b] *Moscow State University, Moscow, 119899 Russia*

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Abstract—Lipid composition, intracellular products of lipid peroxidation (LPO), and the activities of extracellular enzymes were studied during submerged cultivation of the xylotrophic fungus *Lentinus* (*Panus*) *tigrinus* VKM F-3616D. The maximum secretion of ligninolytic enzymes during the phase of active mycelium growth correlated with increased content of readily oxidized phospholipids and unsaturated fatty acids and with low content of the LPO products. In the idiophase, which was characterized by lower excretion of extracellular ligninolytic enzymes, the content of more stable phospholipids, saturated fatty acids, and LPO products increased. A relationship between the composition of mycelial lipids and the secretion of ligninolytic enzymes was revealed.

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Key words: *Lentinus* (*Panus*) *tigrinus*, mycelium, phospholipids, fatty acids, lipid peroxidation.

In recent years, basidiomycetes have attracted ever-increasing interest as organisms possessing unique extracellular ligninolytic and cellulolytic enzyme complexes. These fungi are the only organisms capable of total destruction of lignin and cellulose (the main components of wood), as well as of a great variety of xenobiotics [1, 2]. The fungus *Lentinus* (*Panus*) *tigrinus*, which exhibits high ligninolytic activity, therefore deserves special attention; it can be used for the production of composite materials as well as for the oxidation of phenols and their derivatives [3–6]. The intensity and mechanisms of secretion of ligninolytic enzymes have been shown to depend on the composition of the cytoplasmic membrane, in particular, on the presence of phospholipids (PL). They not only perform barrier functions, but are also involved in transmembrane processes including enzyme translocation across the cytoplasmic membrane [7]. Every growth phase is characterized by a certain ratio of major PL and a by a specific state of the cellular membrane [8]. Moreover, the products of lipid peroxidation (LPO) were shown to have a considerable effect on lignin degradation [9]. The investigation of the relationship between these processes allows both the elucidation of the mechanisms for lipid involvement in the functioning of the extracellular ligninolytic enzyme complex (ELEC) and the regulation of its synthesis and secretion.

The aim of this work was to study the lipid composition, the LPO products, and the ligninolytic activity under submerged cultivation of strain *L. tigrinus* VKM F-3616D.

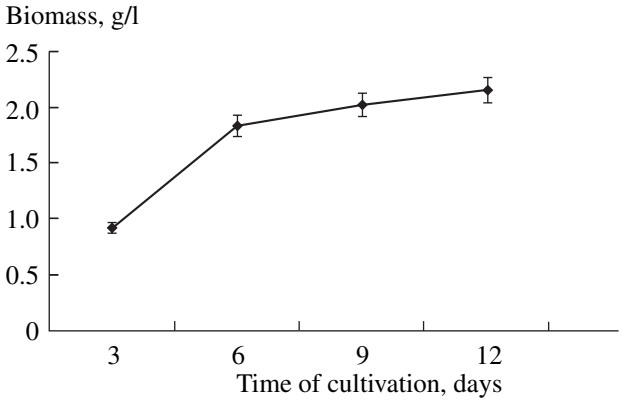
MATERIALS AND METHODS

The ligninolytic fungus *Lentinus tigrinus* VKM F-3616D was isolated at the Biotechnology Department of the Ogarev Mordova State University and deposited with the All-Russia Collection of Microorganisms (VKM) [10].

Inoculum of *L. tigrinus* was cultivated in the Czapek–Dox medium supplemented with 20 g/l (dry weight) of corn extract. A pattern of mycelium grown on wort agar slants (1 × 1 cm) was transferred into 500-ml Erlenmeyer flasks with 100 ml of the medium and cultivated on a thermostated shaker (235 rpm) at 26°C for four days. The lipid composition and enzyme activities were determined on the medium with birch sawdust (20 g/l); the inoculum dose was 5%. Submerged cultivation was performed in shaken (235 rpm) 500-ml Erlenmeyer flasks with 100 ml of the medium at 26°C for 12 days [11].

The lipids were extracted from the mycelium by the Bligh–Dyer method [12]. The composition of phospho-

[1] Corresponding author; e-mail: cadimded@yandex.ru, kistig2@zmail.ru



Time course of biomass accumulation under submerged cultivation of *L. tigrinus*

lipids was analyzed by two-dimensional thin-layer chromatography on silica gel plates. Lipids (300 μg) were streaked onto a plate, which was developed in the Broekhuyse systems: (1) chloroform–methanol–28% ammonia–water (90 : 54 : 5 : 8); (2) chloroform–methanol–glacial acetic acid–water (90 : 40 : 10 : 4) [13]. The spots of phospholipids were visualized by spraying the developed plates with a 5% solution of sulfuric acid in methanol followed by heating at 180°C. The identification of phospholipids was performed with specific reagents [14]. Quantitative estimation of the phospholipids was carried out as described by Vaskovsky and coworkers [15]. The fatty acid methyl esters of total lipids were analyzed by GLC on a Kristall 5000.1 chromatograph (Russia) equipped with an HP-FFAP capillary column (50 m × 0.32 mm) (United States); the programmed temperature range (145– 220°C) was scanned at 4°C/min. Fatty acids were identified by their retention times in relation to that of the internal standard, margaric acid. The amount of dienoic and trienoic conjugates was determined by UV-spectroscopy. Malonic dialdehyde was determined by the thiobarbituric acid (TBA) method [16]. The biomass was determined gravimetrically.

The activities of laccase and peroxidase in the culture liquid were assayed by the oxidation of pyrocatechol and *o*-dianisidine, respectively [17, 18]. The initial reaction rate was measured with an SF-46 (LOMO, Russia) spectrophotometer. One unit of enzyme activity was defined as the amount of the enzyme required for the oxidation of 1 μmol substrate in 1 min under optimal conditions.

All experiments were repeated at least five times. The results were statistically processed using the Microsoft Excel 2000 software package.

RESULTS AND DISCUSSION

Under submerged cultivation, the fungus *L. tigrinus* grew as pellets 1 to 6 mm in diameter. The figure demonstrates that the biomass accumulation was slow. This, to some degree, was the result of the nitrogen limitation created artificially in order to stimulate the ELEC synthesis [19]. The total amount of lipids ranged from 2.6 to 8.0% of dry biomass depending on the growth phase (Table 1). Neutral lipids and phospholipids constituted from 85 to 92 and from 8 to 16% of the total lipids, respectively. These results correlate well with the literature data on the predominance of neutral lipids in the white rot fungi [20]. In the course of cultivation, the amounts of total lipids and neutral lipids increased, whereas the content of the PL fraction decreased.

The PL of *L. tigrinus* consisted of the following fractions: lysophosphatidylcholine + sphingomyelin (LPC + SPM), phosphatidylserine + phosphatidylinositol (PS + PI), phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PEA), and phosphatidylglycerol (PG). The growth of mycelium was accompanied by the changes in the content of individual fractions, whereas the qualitative composition of PL remained unchanged (Table 2). The PEA and PC fractions were the most variable. The phase of active mycelium growth (3–6 days) was characterized by a high content of PEA, which usually incorporated highly unsaturated and readily oxidized fatty acids (FA). In the idiophase (6–12 days), the growth rate decreased and the level of PC increased; this phospholipid is more saturated and oxidation-resistant than other fractions.

The fatty acid composition of the total lipids remained almost unchanged during the fungus growth. The following fatty acids were revealed: palmitic ($C_{16:0}$), PAA), stearic ($C_{18:0}$), oleic ($C_{18:1}$), linoleic ($C_{18:2}$, LA), linolenic ($C_{18:3}$), myristic ($C_{14:0}$), lauric ($C_{12:0}$), arachidonic ($C_{20:4}$), and behenic ($C_{22:0}$) (Table 3). The mycelium of actively growing cultures

Table 1. Changes in the activities of ligninolytic enzymes and the composition of intracellular lipids during the growth of *L. tigrinus*

Time of cultivation, days	Lipids, % of dry mass	Phospholipids, % of the total lipids	Neutral lipids, % of the total lipids	Peroxidase, U/ml	Laccase, U/ml
3	2.6 ± 0.1	15.7 ± 0.6	84.3 ± 4.3	0.111 ± 0.006	5.919 ± 0.296
6	4.6 ± 0.2	9.3 ± 0.4	90.7 ± 4.2	0.922 ± 0.046	22.559 ± 1.128
9	5.8 ± 0.2	8.5 ± 0.4	91.5 ± 4.8	0.541 ± 0.027	8.099 ± 0.404
12	8.0 ± 0.2	7.8 ± 0.4	92.2 ± 4.5	0.221 ± 0.011	1.519 ± 0.076

Table 2. Changes in the composition of intracellular phospholipids (% of the sum) during growth of *L. tigrinus*

Time of cultivation, days	PG	PEA	PA	PC	PS + PI	LPC + SPM
3	13.8 ± 0.7	42.7 ± 2.1	5.9 ± 0.2	15.9 ± 0.7	20.8 ± 1.0	0.9 ± 0.1
6	9.8 ± 0.5	29.4 ± 1.4	5.9 ± 0.2	32.0 ± 1.6	20.6 ± 0.9	2.3 ± 0.2
9	9.3 ± 0.4	21.7 ± 1.1	6.6 ± 0.3	38.8 ± 1.9	20.9 ± 0.9	2.7 ± 0.2
12	8.5 ± 0.4	20.8 ± 1.1	6.8 ± 0.4	39.5 ± 1.9	19.9 ± 0.8	4.5 ± 0.4

Table 3. The fatty acid composition of the total lipids during growth of *L. tigrinus*

Fatty acids	Fatty acids (μg/mg lipids)			
	3 days	6 days	9 days	12 days
$C_{12:0}$	0.42 ± 0.02	0.34 ± 0.02	0.13 ± 0.01	0.15 ± 0.01
$C_{14:0}$	1.86 ± 0.09	2.30 ± 0.12	1.26 ± 0.06	1.24 ± 0.06
$C_{16:0}$	9.00 ± 0.45	17.20 ± 0.86	16.74 ± 0.84	18.9 ± 0.95
$C_{18:0}$	2.05 ± 0.10	6.10 ± 0.31	8.93 ± 0.45	10.2 ± 0.51
$C_{18:1}$	4.07 ± 0.20	8.26 ± 0.41	9.96 ± 0.50	10.1 ± 0.51
$C_{18:2}$	10.92 ± 0.55	19.69 ± 0.98	11.97 ± 0.60	7.3 ± 0.37
$C_{18:3}$	0.54 ± 0.03	3.45 ± 0.17	0.49 ± 0.02	0.38 ± 0.02
$C_{20:4}$	0.24 ± 0.01	0.10 ± 0.01	–	–
$C_{22:0}$	0.08 ± 0.01	0.48 ± 0.02	0.45 ± 0.02	0.50 ± 0.03
Saturated	13.41 ± 0.67	26.42 ± 1.32	27.51 ± 1.38	30.99 ± 1.55
Unsaturated	15.77 ± 0.79	31.50 ± 1.58	22.42 ± 1.12	17.78 ± 0.89
Coefficient of unsaturation	1.17 ± 0.06	1.20 ± 0.06	0.81 ± 0.04	0.57 ± 0.03

(3–6 days) contained high amounts of unsaturated fatty acids with predominance of LA (over 40%); the main saturated fatty acid was PAA (over 30%); the coefficient of unsaturation reached the maximum value (1.17–1.20). In the idiophase, the level of unsaturated fatty acids decreased, and the coefficient of lipid unsaturation was as low as 0.81–0.57.

The dynamics of laccase and peroxidase activities during mycelium growth were similar (Table 1). The activities of both enzymes increased in the trophophase (3–6 days), reaching the maximal values after six days of cultivation, and decreased considerably in the idiophase. A certain correlation was revealed between variations in the enzyme activities and the lipid composition of the mycelium. The maximal synthesis and secretion of ELEC correlated with the predominance of readily oxidized PL containing a high amount of unsaturated fatty acids and therefore low microviscosity of the membranes [8]. A decrease in the synthesis and secretion of ELEC correlated with an increase in the level of more stable PL and a decrease in the content of unsaturated fatty acids, mainly LA.

The LA is considered to play a special role in fungal metabolism; in particular, the white rot fungi were shown to have a higher content of LA than other xylotrophic fungi; this result is in agreement with our data [21]. In the course of lignin degradation, these fungi generated reactive oxygen species in order to activate lipid peroxidation; its intensity is determined by the presence of unsaturated fatty acids, primarily LA. The peroxide radicals can then be involved in lignin destruction both directly and through the activation of ligninolytic enzymes [9]. High levels of lipid peroxidation could have been expected in our experiments, since the medium included lignin-containing birch sawdust and the amount of unsaturated fatty acids increased in the trophophase. However, in this growth phase, intrac-

Table 4. Changes in the content of the LPO products during growth of *L. tigrinus*

Time of cultivation, days	Dienoic conjugates, U/mg lipids	Trienoic conjugates, U/mg lipids	Malonic dialdehyde, nmol/mg biomass
3	26.4 ± 1.3	24.2 ± 1.2	6.2 ± 0.3
6	24.5 ± 1.2	15.7 ± 0.7	11.9 ± 0.5
9	42.3 ± 2.1	30.8 ± 1.5	33.8 ± 1.6
12	48.1 ± 2.4	36.2 ± 1.8	52.8 ± 2.6

ellular content of dienoic and trienoic conjugates, primary LPO products, was relatively low (Table 4). In the idiophase, the amount of dienoic and trienoic conjugates increased sharply, although the level of readily oxidized lipids decreased, probably due to the decreased amount of natural bioantioxidants in membranous lipids [8]; consequently, the cells became unable to regulate the level of LPO products adequately. An increase in the level of more stable lipids in the idiophase is a conceivable mechanism for the maintenance of a stable level of oxidative reactions in the membranous lipids even at the expense of the decreased intensity of metabolic processes. The amount of malonic dialdehyde increased throughout the fungus cultivation. The results obtained indicate a certain discrepancy between the accumulation of the primary (dienoic and trienoic conjugates) and secondary LPO products. A plausible explanation for this is that hydroperoxides (determined by UV absorption) mainly characterize the oxidation of unsaturated fatty acids, whereas TBA-reactive products are formed in the reactions not only with malonic dialdehyde, but also with other metabolites, such as its precursors, aldehydes and aldehyde acids formed via the cleavage of the hydrocarbon chains of the fatty acid hydroperoxides [22]. That is why a linear correlation between the accumulation of primary and secondary LPO products it is not always observed.

Thus, a relationship between the ELEC secretion and the lipid composition of *L. tigrinus* mycelium was revealed. The phase of intense ELEC synthesis was characterized by increased content of readily oxidized PL, such as PEA, PI, and PS, and unsaturated fatty acids and low amounts of LPO products. The transition of mycelium to the idiophase is accompanied by an increase in the level of PC and saturated fatty acids.

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