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ORIGINAL PAPER

Effect of aromatic compounds on the production of laccase and manganese peroxidase by white-rot basidiomycetes

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Abstract Three white-rot fungi displayed a wide diversity in their response to supplemented aromatic compounds. Pyrogallol stimulated *Cerrena unicolor* laccase and manganese peroxidase (MnP) synthesis in synthetic medium 2.5- and 2-fold, respectively, whereas 2,4,6-trinitrotoluene (TNT) brought about a 2.8-fold increase in laccase yield by *Trametes versicolor* in submerged fermentation of ethanol production residue. No effect of the tested aromatic compounds on enzyme secretion by *Ganoderma lucidum* in mannitol-containing medium was detected. Nevertheless, *G. lucidum* is a potent producer of laccase in submerged fermentation of wheat bran and enzyme synthesis can be further increased by supplementation of medium with an appropriate inducer. The structure and the concentration of aromatic compounds play an important role in the regulation of enzyme synthesis. The supplementation of synthetic medium with 0.03–0.3 mM TNT or hydroquinone increased the differential rate of laccase synthesis by *C. unicolor* from 1,267 to 3,125–8,630 U mg biomass^{-1} day^{-1}. Moreover, the same aromatic compound may function as either an inducer or a repressor, depending on the fungus and enzyme studied. Thus, hydroquinone increased 3-fold *T. versicolor* laccase activity decreasing 2- and 8-fold the yields of MnP and endoglucanase, respectively.

Keywords White-rot basidiomycetes · Aromatic compounds · Laccase · Manganese peroxidase · Production

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Introduction

Basidiomycetes represent a taxonomically, ecologically, and physiologically extremely diverse group of eukaryotic organisms. White-rot basidiomycetes are investigated extensively because of their unique ligninolytic system that includes laccases and peroxidases as important biocatalysts for their application in bioremediation, pulp and paper, textile, dye, and food industries. All these biotechnological applications require large amounts of low-cost enzymes. Therefore, to develop commercially significant technologies of ligninolytic enzyme production the major task is to extend the spectrum of producing organisms and comprehensively investigate the physiological mechanisms regulating and enhancing enzyme synthesis.

One of the most effective approaches to increase the yield of ligninolytic enzymes is the supplementation of the nutrient medium with an appropriate inducer. The most widely reported effective inducer of laccase synthesis is 2,5-xylidine [2, 6, 14]. However, laccase production by *T. versicolor* doubled when veratryl alcohol or guaiacol was used instead of 2,5-xylidine [11]. The best inducer for the *Lentinus strigosus* laccase synthesis is reported to be 2,6-dimethoxyphenol elevating the enzyme yield 8-fold compared to the control, whereas veratryl alcohol was inefficient for the cultures studied or induced the enzyme production insignificantly [13]. This means that the fungus-specific inducer should be found in order to maximally express the target enzyme secretion. Moreover, comparative studies on both laccase and MnP induction in synthetic and lignocellulose containing media are scarce. At the same time, the utilization of some plant raw materials provides an opportunity to produce especially high yields of enzymes without supplementation of the culture medium with specific inducers [7, 8].

In the present work, we tried to evaluate a response of three taxonomically and physiologically different white-rot basidiomycetes on the effect of several widely used and new aromatic compounds. The time course of target enzyme accumulation was studied to analyze the peculiarities of enzyme secretion by these fungi and put forward possible strategies to improve enzyme production in future studies. Moreover, the significance of nutrient medium composition for the enzyme activity expression was also assessed and the cooperative effect of chemical inducers with natural stimulators on laccase production was investigated.

Materials and methods

Organisms and inoculum preparation

The white-rot fungi *Cerrena unicolor* IBB 300, *Ganoderma lucidum* IBB 447, and *Trametes versicolor* IBB 775 were used in this study. Fungal inocula were prepared by growing the strains on a rotary shaker at 150 rpm and 27°C in 250-ml flasks containing 100 ml of the following standard medium (g l^{-1}): glucose 10, NH_4NO_3 1, KH_2PO_4 0.8, K_2HPO_4 0.6, $MgSO_4 \cdot 7H_2O$ 0.5, yeast extract 2. After 7 days of cultivation, mycelial pellets have been homogenized with a Waring laboratory blender.

Cultivation conditions

Mycelial homogenates (4 ml) were used to inoculate the 250-ml flasks containing 50 ml of the following growth medium (g l^{-1}): ammonium tartrate 2, KH_2PO_4 0.8, K_2HPO_4 0.6, $MgSO_4 \cdot 7H_2O$ 0.5, $CuSO_4 \cdot 5H_2O$ 0.1 mM, $MnSO_4 \cdot 5H_2O$ 0.1 mM, yeast extract 3. Mannitol (10 g l^{-1}), wheat bran (best growth substrate for the enzyme production by *G. lucidum*) and residue after ethanol production from wheat grain (best growth substrate for the enzyme production by *C. unicolor* and *T. versicolor*) (both 40 g l^{-1}) served as growth substrates. The initial pH of all media was adjusted to 6.0 prior to sterilization. In order to study the effect of inducers on enzyme production, various aromatic compounds were added to the medium from the beginning of cultivation to give a final concentration of 0.5 mM. Media without aromatic compounds served as control. In parallel with long-term experiments, short-term cultivations have been carried out in the study of the effects of TNT (2,4,6-trinitrotoluene) and 1,4-hydroquinone concentration on enzyme production. In this specific case, the homogenized mycelium of *C. unicolor* was inoculated at concentration of 2 ± 0.1 g l^{-1} in the synthetic medium containing 1% mannitol and after 20 h of cultivation 25 ml of small pellets were inoculated in 250-ml flasks containing 25 ml growth medium (consisting of double concentration of all ingredients). Initial concentration of the inoculated biomass was 1.3 ± 0.1 g l^{-1}.

All fungi were cultivated on a rotary shaker at 150 rpm and 27°C. At given time intervals, samples (1 ml) were taken and the solids were separated by centrifugation at 13,000 × *g* for 5 min at 4°C. The supernatants were analyzed for enzyme activity.

Analytical methods

Fungal biomass was measured gravimetrically after recovering mycelium by centrifugation of whole cultures at 8,000 × *g* for 15 min and drying at 70°C for 24 h.

Laccase (EC 1.10.3.2) activity was determined by monitoring the absorbance change at 420 nm related to the rate of oxidation of 1 mM 2,2′-azino-bis-[3-ethylbenzthiazoline-6-sulfonate] (Sigma®) to its cation radical in 25 mM Na-acetate buffer (pH 3.8) at room temperature [3]. MnP (EC 1.11.1.13) activity was measured at 270 nm by following the formation of Mn^{3+}-malonate-complexes [20]. One unit of laccase or MnP activity was defined as the amount of enzyme that leads to the oxidation of 1 μmol of substrate per minute.

Endoglucanase (EG) (EC 3.2.1.) activity was determined in accordance with the International Union of Pure and Applied Chemistry recommendations by mixing 70 μl appropriately diluted sample with 630 μl 1% carboxymethyl cellulose (sodium salt, low viscosity, Sigma®) in 50 mM citrate buffer (pH 5.0) at 50°C for 10 min [9]. Glucose standard curves were used to calculate the cellulase activity. Release of glucose was measured using the dinitrosalicylic acid reagent method [12]. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of glucose per minute.

All experiments were performed at least twice using three to five replicates. The data presented correspond to mean values, the standard deviation being lower than 15%.

Results

Effect of aromatic compounds on ligninolytic enzyme production

When *C. unicolor* was grown in the synthetic mannitol-containing medium, the fungus grew well in the presence of eight aromatic compounds (Table 1). However, total growth inhibition of this fungus occurred when 0.5 mM of either hydroquinone or TNT was added in the medium.

Pyrogallol followed by 2,6-DMP (dimethoxyphenol), catechol, and veratric acid caused between 1.6 and 2.5-fold increase of laccase activity as compared to the control

Table 1 Effect of aromatic compounds on *C. unicolor* enzyme activity

Compounds	Mannitol-containing medium			EPR[a]-containing medium		
	Biomass (mg ml^{-1})	Laccase (U ml^{-1})	MnP (U ml^{-1})	Laccase (U ml^{-1})	MnP (U ml^{-1})	CMCase (U ml^{-1})
Control	7.9 ± 0.5	15.0 ± 1.4	2.0 ± 0.2	117 ± 14	4.1 ± 0.3	58 ± 5
Catechol	7.1 ± 0.8	28.6 ± 2.5	2.4 ± 0.2	114 ± 9	4.8 ± 0.5	44 ± 5
2,6-DMP[a]	8.4 ± 0.7	29.7 ± 3.7	2.3 ± 0.3	137 ± 13	8.5 ± 1.1	54 ± 6
Ferulic acid	7.5 ± 0.6	15.1 ± 1.3	2.6 ± 0.2	118 ± 10	6.6 ± 0.6	44 ± 6
Hydroquinone	0.2 ± 0	0	0	94 ± 14	3.6 ± 0.4	2 ± 1
Pyrogallol	7.9 ± 0.8	37.2 ± 3.4	3.9 ± 0.3	153 ± 13	6.5 ± 0.7	55 ± 4
TNT[a]	0.2 ± 0	1.7 ± 0.2	0	165 ± 18	4.1 ± 0.6	28 ± 4
Vanillin	7.4 ± 0.6	2.9 ± 0.3	2.3 ± 0.2	170 ± 22	5.8 ± 0.6	24 ± 3
Vanillic acid	8.2 ± 0.6	3.1 ± 0.3	1.4 ± 0.2	154 ± 15	7.4 ± 0.9	39 ± 3
Veratric acid	8.5 ± 0.7	24.1 ± 3.0	3.2 ± 0.4	83 ± 9	6.4 ± 9.6	66 ± 8
Xylidine	7.1 ± 0.8	14.2 ± 2.1	0.9 ± 0.1	103 ± 12	4.2 ± 0.4	41 ± 4

[a] *EPR* ethanol production residue, *DMP* dimethoxyphenol, *TNT* 2,4,6-trinitrotoluene

culture. It is worth noting that in all laccase accumulating cultures enzyme activity was detected on day 3, aromatic compounds accelerated an enzyme production and shortened the time of peak activity appearance. Moreover, veratric acid and pyrogallol stimulated 1.6- and 2-fold, respectively, MnP production by *C. unicolor*. None of the other aromatic compounds tested showed appreciable increase of laccase or MnP activity. On the contrary, vanillin and vanillic acid significantly repressed laccase synthesis, whereas xylidine and vanillic acid retained MnP accumulation. It is worth noting that in spite of complete inhibition of *C. unicolor* growth by TNT, significant laccase activity was observed (1.7 U ml^{-1} on day 11) proving that an inoculated mycelium remained metabolically active.

When *C. unicolor* was cultivated in medium containing EPR, a lignocellulosic growth substrate found most appropriate for this fungus, the growth inhibition in the presence of TNT was observed during the first 4–5 days. Afterwards, small pellets were seen and active synthesis of enzyme occurred. Vanillin followed by TNT, vanillic acid, and pyrogallol at a concentration of 0.5 mM increased the laccase yield from 117 to 153–170 U ml^{-1} (Table 1). Under the same conditions, laccase activity in veratric acid and hydroquinone supplemented cultures was rather lower than that recorded in control medium. The supplementation of 2,6-DMP to the control medium increased the MnP yield by two-fold. Vanillic acid followed by ferulic acid, pyrogallol, and veratric acid increased *C. unicolor* MnP activity by more than 50%. Among the compounds tested, only hydroquinone repressed enzyme production under the same cultivation conditions.

Cultivation of *T. versicolor* in EPR-containing medium for 14 days in the presence of xylidine and hydroquinone resulted in, respectively, 2- and 2.8-fold higher extracellular laccase levels as compared to the control medium (Table 2). None of the other compounds tested had any significant effect on laccase secretion from this fungus. Conversely, all the tested aromatic compounds reduced MnP levels when present in the culture medium. This effect was more pronounced for catechol, 2,6-DMP, ferulic and veratric acids.

Table 2 Effect of aromatic compounds on *T. versicolor* enzyme activity in submerged fermentation of EPR

Compounds	Laccase (U ml^{-1})	MnP (U ml^{-1})	CMCase (U ml^{-1})
Control	7.1 ± 0.5	0.6 ± 0.08	44 ± 3
Catechol	8.2 ± 0.8	0.1 ± 0.02	45 ± 5
2,6-DMP	6.9 ± 0.5	0.1 ± 0.01	47 ± 4
Ferulic acid	8.4 ± 9	0.1 ± 0.01	45 ± 3
Hydroquinone	19.7 ± 2.3	0.3 ± 0.02	6 ± 1
Pyrogallol	6.7 ± 0.8	0.2 ± 0.02	22 ± 3
TNT	9.0 ± 1.4	0.2 ± 0.03	18 ± 3
Veratric acid	6.7 ± 0.8	0.1 ± 0.01	43 ± 4
Xylidine	14.5 ± 2.0	0.2 ± 0.03	33 ± 3

Recently, we have shown that in submerged cultivation in synthetic glucose-containing medium, *G. lucidum* produces only traces of laccase activity [15]. To increase the laccase secretion by this fungus, several aromatic compounds were tested as potential enzyme synthesis inducers. Upon cultivation in mannitol-containing medium, neither fungal growth nor laccase production was significantly affected by supplementation of the nutrient medium with aromatic compounds, with the exception of TNT (Table 3). Moreover, no MnP activity was seen in cultures with or without added aromatic compounds. In submerged fermentation of wheat bran, *G. lucidum* accumulated significant levels of laccase activity. However, among aromatic compounds used, only the addition of 2,6-DMP or pyrogallol enhanced *G. lucidum* laccase activity by 20–30% as

Table 3 Effect of aromatic compounds on *G. lucidum* enzyme activity

Compounds	Mannitol-containing medium		Wheat bran-containing medium	
	Biomass (mg ml^{-1})	Laccase (U ml^{-1})	Laccase (U ml^{-1})	CMCase (U ml^{-1})
Control	4.3 ± 0.4	0.12 ± 0.01	71 ± 8	1.8 ± 0.1
2,6-DMP	4.4 ± 0.3	0.08 ± 0.01	88 ± 7	2.2 ± 0.2
Ferulic acid	4.6 ± 0.5	0.14 ± 0.02	76 ± 10	2.0 ± 0.2
Pyrogallol	4.8 ± 0.5	0.10 ± 0.01	94 ± 10	1.6 ± 0.2
TNT	0.2 ± 0	0.03 ± 0.01	43 ± 6	1.9 ± 0.3
Xylidine	4.7 ± 0.5	0.12 ± 0.01	69 ± 7	1.5 ± 0.2

compared to the control without exogenous aromatic compound.

Effect of aromatic compounds on endoglucanase production

Data have been published [1, 5, 18, 19] indicating that phenolic compounds may greatly affect the synthesis and activity of cellulases and xylanases in mycelial fungi. The capacity of fungi to produce high levels of hydrolases is of importance in supplying the growing cultures with a carbon source essential for their biosynthetic activity. To examine the specificity of aromatic compounds effect on enzyme secretion by selected fungi in the presence of 0.5 mM aromatic compounds, basidiomycetes CMCase activity was also measured in fermentation of lignocellulose. Among the compounds tested, hydroquinone appeared to be the most potent repressor of enzyme synthesis by *C. unicolor* (Table 1) and *T. versicolor* (Table 2) decreasing the endoglucanase activity 29- and 7-fold, respectively, as compared to the control media. Vanillin and TNT in the case of *C. unicolor*, as well as TNT and pyrogallol in the case of *T. versicolor* two-fold lowered the CMCase yields. Nevertheless, even comparatively low enzyme activity appeared to be sufficient to ensure significant growth of fungi and laccase production. Moreover, veratric acid supplemented to the control medium caused a 50% increase of *C. unicolor* CMCase yield. In contrast to these fungi, none of the tested aromatic compounds significantly affected the CMCase production by *G. lucidum.*

Effect of TNT and hydroquinone concentration

Our data showed that TNT and hydroquinone at a concentration of 0.5 mM completely suppressed growth of *C. unicolor* and *G. lucidum* in cultivation in synthetic medium. However, in lignocellulose-containing media, these compounds rather stimulated laccase production by *C. unicolor* and *T. versicolor.* Therefore, it was reasonable to study the target enzyme production as a function of TNT and hydroquinone concentration. Results of short-term experiments presented in Table 4 indicate that concentrations between 0.03 and 0.1 mM of TNT and 0.03 mM of hydroquinone did not affect fungal growth. However, higher concentrations of these compounds delayed the biomass accumulation during the first 24–48 h of submerged cultivation. Afterwards, the fungal growth in these media accelerated and by 96 h the biomass yields were comparable to that in the control medium. The fungus short-term cultivation showed that both aromatic compounds highly accelerated laccase production increasing already after 8 h the enzyme activity between 3- and 5-fold as compared to the control

Table 4 Effect of inducer concentration on the *C. unicolor* biomass accumulation and differential rate of laccase synthesis in mannitol-containing medium

Compounds	mM	Biomass[a] (g l^{-1})				DRLS[b] (dU/dg/day)
		Cultivation time (h)				
		8	24	48	96	24–48
Control	0	1.6 ± 0.1	2.3 ± 0.1	3.3 ± 0.2	5.0 ± 0.2	1,267
TNT	0.03	1.6 ± 0.1	2.3 ± 0.1	3.5 ± 0.2	4.9 ± 0.2	3,125
	0.1	1.5 ± 0.1	2.3 ± 0.2	3.4 ± 0.3	4.8 ± 0.2	3,473
	0.3	1.3 ± 0.1	1.5 ± 0.1	2.5 ± 0.3	4.8 ± 0.3	8,630
Hydroquinone	0.03	1.5 ± 0.1	2.2 ± 0.1	3.2 ± 0.3	4.9 ± 0.3	4,330
	0.1	1.3 ± 0.1	1.5 ± 0.1	2.4 ± 0.3	4.6 ± 0.3	6,522
	0.3	1.2 ± 0.1	1.2 ± 0.1	1.6 ± 0.2	4.6 ± 0.3	5,900

[a] Initial biomass concentration was 1.3 g l^{-1}

[b] *DRLS* differential rate of laccase synthesis

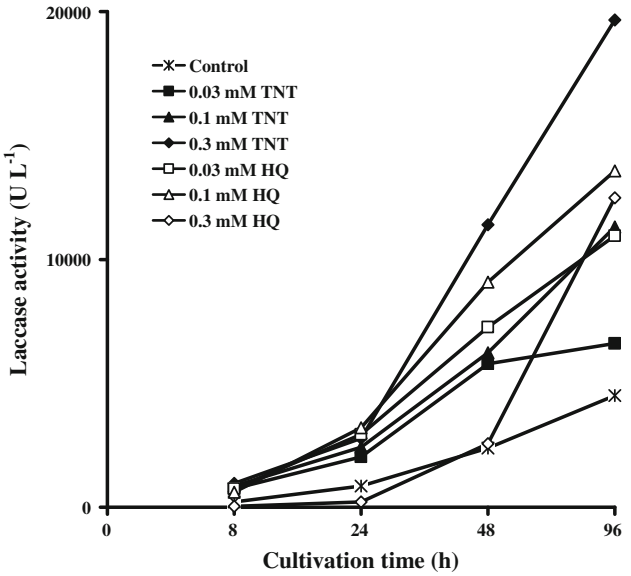

Fig. 1 Effect of TNT and hydroquinone (HQ) concentration on *Cerrena unicolor* laccase production in mannitol-containing medium

medium without an inducer (Fig. 1). During the second day of fungus growth, the differential rate of laccase synthesis in the presence of TNT reached 3,125–8,630 U g biomass^{-1}, significantly exceeding that in the control medium (Table 4). Only 0.3 mM hydroquinone completely repressed enzyme production during the first day of cultivation. However, after 24 h, laccase synthesis started with the differential rate reaching 5,900 U g biomass^{-1} during the second day of growth and after 4 days of submerged cultivation in this medium the laccase yield was 2.8-fold higher as compared with the control variant.

Discussion

Detailed information on the regulation of ligninolytic enzyme synthesis is essential for the development of cost-effective technologies of these enzymes' production and application. In this study, a distinct effect of some aromatic compounds on the laccase and MnP production by *C. unicolor* and *T. versicolor* was revealed. An especially potent inducer for both laccase and MnP synthesis by *C. unicolor* in synthetic medium appeared to be pyrogallol, whereas TNT strongly increased *T. versicolor* laccase yield in submerged fermentation of EPR. Some of the aromatic compounds used in the lignocellulose fermentation by *T. versicolor* and *G. lucidum* had a significant influence on the laccase production, but all of them failed to induce MnP synthesis. In contrast to observed *Pycnoporus cinnabarinus* [10] and *Pleurotus pulmonarius* [16] laccase synthesis induction by ferulic acid, no effect of this compound was revealed in our study. Moreover, in contrast to many other reports [2, 6, 14], xylidine did not promote laccase synthesis by *C. unicolor* and *G. lucidum.* Furthermore, no effect of the tested aromatic compounds on *G. lucidum* enzyme activity was detected in synthetic medium.

Several conclusions arise from these results. White-rot fungi display a wide diversity in their response to enzyme inducers. Firstly, these and other results [13] suggest that enhancement of laccase and MnP production in response to various aromatic compounds depends on fungal physiological, genetic, or ecological peculiarities. Thus, *G. lucidum* IBB 447 in contrast to *G. lucidum* FP-58537-Sp [4], *C. unicolor*, and *T. versicolor* produces only traces of laccase activity in synthetic medium. Moreover, aromatic compounds tested did not induce enzyme synthesis by this strain. Nevertheless, *G. lucidum* IBB 447 is a potent producer of laccase in submerged fermentation of wheat bran and enzyme synthesis can be further increased by the supplementation of medium with an appropriate inducer. This illustrates the need to adapt a screening program using various media and inducers in a manner that would not lead to prematurely discarding fungi. Secondly, there is no aromatic/phenolic compound serving as universal enzyme synthesis inducer. However, the same compound (such as pyrogallol in *C. unicolor* cultivation in mannitol-containing medium) may stimulate simultaneous laccase and MnP production. Moreover, the same aromatic compound may function as either an inducer or a repressor, depending on the fungus and enzyme tested. Thus, in submerged fermentation of EPR by *T. versicolor* hydroquinone 3-fold increased production of laccase decreasing 2-fold and 8-fold MnP and CMCase yields, respectively, as compared to the control medium.

Our results show that not only the structure of aromatic compounds but also their concentration plays an important role in the regulation of enzyme synthesis. This finding is consistent with an earlier report by Xiao et al. [21] who showed that the laccase activity of *Trametes* sp. AH28-2 dramatically increased along with the increase of guaiacol, *o*-toluidine, and 3,5-dihydroxytoluene concentration from 0.5 mM to as high as 12, 4, and 20 mM, respectively. In this study, the calculation of *C. unicolor*-specific laccase activities showed that both TNT and hydroquinone induced enzyme production since the specific laccase activity after 8 h of fungus cultivation increased from 131 U g^{-1} biomass in the control medium to 462, 573, and 746 U g^{-1} biomass with elevation TNT concentration to 0.03, 0.1, and 0.3 mM. Supplementation of control medium with 0.03 and 0.1 mM hydroquinone caused after 24 h *C. unicolor* cultivation the increase of specific laccase activity from 374 U g^{-1} to 1,332 and 2,140 U g^{-1}. Moreover, even in medium containing 0.3 mM hydroquinone the enzyme production greatly accelerated after 24 h of cultivation (Fig. 1). Furthermore, in contrast to other studies, the differential rates of laccase

synthesis by *C. unicolor* were determined. The data obtained prove that the fungus cultivation in 0.03–0.3 mM TNT or hydroquinone-containing media increased the differential rates of laccase synthesis 2.5–6.8-fold and 3.4–5.2-fold, respectively, as compared to that in the control medium (Table 4).

However, why do structurally very different compounds effectively induce laccase? Induction of laccase by aromatic compounds has been theorized to constitute a protective response of fungi to the presence of potentially toxic compounds produced during the degradation of the lignin [16, 17]. In fact, the overproduction of laccase activity by *Trametes* sp. AH28-2 was revealed at extremely high concentrations (4–20 mM) of aromatic compounds [21]. In this study, TNT in a concentration of 0.5 mM completely inhibited *C. unicolor* growth in a synthetic medium; nevertheless, the resting mycelium secreted a significant level of laccase activity (Table 1). Moreover, 0.3 mM TNT and 0.1–0.3 mM hydroquinone became toxic to *C. unicolor* grown in mannitol-containing medium (Table 4). However, at these exact concentrations, these compounds provided the highest differential rates of laccase synthesis. Hence, one could suggest that enhanced laccase synthesis may function as a defense mechanism against chemical stress.

Another possible explanation is that compounds with different substituted groups can stimulate synthesis of different laccase isozymes [16, 21]. Finally, the inducing effect of a new compound appearing as result of transformation/degradation of supplemented aromatic compound cannot be ruled out. Elucidation of such compounds stimulating ligninolytic enzyme synthesis by selected basidiomycetes is under investigation.

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