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Assessment of laccase activity synthesized by Basidiomycota fungi in the presence of wood impregnated with creosote oil

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Abstract: *Assessment of laccase activity synthesized by Basidiomycota fungi in the presence of wood impregnated with creosote oil.* The article presents the results of the assessment of laccase activity, synthesized by fungi causing white decomposition of wood in the presence of wood samples impregnated with creosote oil in the growth environment. The obtained results indicate that the creosote oil contained in wood modulates the activity of this enzyme. Creosote oil definitely stimulates the cells of the T. versicolor fungus to induce laccase synthesis, which may be of great practical importance in terms of the possibility of designing a biotechnological method of biodegradation of pollutants, including contaminants in the form of impregnated wood waste

Keywords: Basidiomycota, creosote oil, laccase

INTRODUCTION

Creosote oil is a mixture of polycyclic aromatic compounds with strong biocidal activity. It contains neutral compounds, such as anthracene, naphthalene, and acid and basic components. The substances included in the creosote oil have strong toxic properties in relation to higher organisms. One of the main components of creosote is benzo[a]pyrene, which is highly cytotoxic and immunotoxic (Zasadowski and Wysocki 2002). Due to its toxic properties, creosote oil is intended only for the impregnation of wood used in places where other biocidal preparations would not provide sufficient effectiveness in the assumed life cycle of the product - impregnated wood. It is assumed that the fungicidal value of creosote oil is from 8.8 to 12.9 kg/m^3, and the durability of wood protected with this impregnation may reach about 30 years under normal conditions. Some literature data indicate that the service life of wood saturated with impregnating oil can be up to 80-90 years. The level of toxicity of creosote oil in impregnated wood is almost unchanged, and the initiation of degradation of properly impregnated wood by biotic factors may occur when the impregnate content is reduced as a result of weather conditions (Broese van Groenou *et al.* 1951).

Handling firewood, impregnated with creosote oil, requires proper disposal, most often in the form of storage in a properly secured landfill. However, it should be noted that in the era of very restrictive waste regulations, many effective and safe methods of neutralization of hazardous substances or products are sought. In the literature for years, you can find experimental works presenting the results of biotechnological methods of utilization of creosote oil (Cerniglia, 1997, Atagana *et al.* 2006). The potential for bioremediation of various types of contaminants by bacteria and fungi has been described by numerous authors (Daane *et al.* 2002, Ghosal *et al.* 2016, Smułek *et al*. 2020). The ability of fungi to decompose substances contained in oil tar was analyzed by Kumar and Kaur (2018). Ren *et al.* (2018), showed that bacterial cells that show a higher affinity for aromatic hydrocarbons can use these compounds as a carbon source. Particular importance in the neutralization of substances contained in creosote oil is attributed to the fungi that cause white decomposition of wood (Lamar *et al.*, 2002). It should be noted that, on the one hand, wood impregnation is to protect the wood against decomposition caused by fungi, and on the other hand, fungi can potentially be a source of biologically active

substances that allow the neutralization of harmful substances contained in the impregnated wood. The effectiveness of neutralization of xenobiotic compounds by white wood decomposition fungi is attributed to the activity of redox enzymes, especially laccase and Mn-dependent peroxidase. There are many scientific studies indicating the potential use of laccase in the neutralization of pesticides (Pozdnyakova *et al.* 2001) or polycyclic aromatic hydrocarbons (Bressler *et al.* 2000). Bollag and Myers (1992) investigated the effect of *Trametes hirsuta* on the degradation of alkenes, while *Coriolopsis gallica* laccase used for the utilization of carbozole, N-ethylcarbozole, fl uorine, and dibenzothiophene was the subject of research by Dec and Bollag (2000). Based on the data presented in the literature, it can be concluded that the laccase enzyme can be used in the utilization of polycyclic aromatic hydrocarbons through their oxidation into less toxic compounds (Mayer and Staples 2002). Borras *et al.* (2010) showed that the laccase synthesized by *T. versicolor* led to the complete oxidation of anthracene and naphthalene. In other studies by Stella et al. (2012) demonstrated the ability of the fungus *Lentinus tigrinus* to remove chlorobenzoic acid from the environment through an efficient system of intracellular and extracellular enzymes (including Mn-preoxidase laccase) synthesized by fungi.

The paper presents the results of research on changes in the activity of the laccase enzyme synthesized by *Trametes versicolor, Armillaria borealis* and *Pleurotus ostreatus* in the presence of wood impregnated with creosote oil. The research results indicate that the presence of the biocide induces the synthesis of highly active laccase, especially by the fungus *T. versicolor*. The study showed that laccase activity depends on the amount of impregnated wood added to the fungal growth medium.

MATERIALS AND METHODS

The laccase-synthesizing fungi came from the cultures collection of the Department of Wood Science and Wood Protection, Warsaw University of Life Sciences. Fungi starter cultures *Trametes versicolor* (L.) Lloyd strain 30, *Armillaria borealis* Marxm. & Korhonen strain 01 and *Pleurotus ostreatus* (Jacq.) P. Kumm (n.d.) After this time, the fungal cultures were transplanted onto liquid mineral media, with each fungus species being inoculated on a different mineral medium. The choice of the culture medium was dictated by the expected high enzymatic activity of fungi on given culture media (Żuchwoski *et. al.* 2015, Jaszek *et al.* 2006). The enzymatic activity tests were carried out on a mineral liquid medium according to Fahreus for *T. versicolor* and *A. borealis* as well as Lindeberg's and Czapek medium for *P. ostreatus*. Starting colonies were homogenized using sterile glass beads. Then 250 µl of homogenate (mycelial inoculum) was taken from each culture and added to 100 ml of the appropriate medium. Cultures were carried out in a heat incubator for 10 days, in conditions of temperature and relative air humidity, which were 26 ± 2 ° C and 66 ± 2%. Samples of wood impregnated with creosote oil, meeting the standards contained in PN-C-97023: 1983, were obtained from the "Nasycalnia Podkładów CZEREMCHA". These samples were ground using a laboratory knife mill to form particles that were 1.2 to 1.5 mm in size. The control wood samples were prepared in the same way. Ten days after inoculation, a suitable proportion of crushed pine wood impregnated with creosote oil was added to each flask. The control samples contained identical weights of fragmented wood, but devoid of the biocidal agent. The number of individual samples of wood added to the flasks with mycelium is presented in Table 1.

The wood samples added to the medium with mycelium were sterilized by radiation. The radiation parameters of the samples were as follows: dose 28 kGy, transporter 0.471 m/min, set current 520 mA. The fungi were grown in a heat incubator. The conditions for the incubation of the fungi on the substrates with the addition of wood particles containing creosote oil did not change

Table 1. Chipped wood content in different variants of the experiment

Sample code	Amount of chopped wood/100 ml of nutrient solution	Type of samples
KBz	-	Treatment-free control - mycelium growing on a liquid culture medium without the addition of chopped wood
KZ-1	0.1g	Treatment control - mycelium growing on a liquid culture medium with the addition of crushed wood
KZ-2	0.25g	
KZ-3	0.5g	
B-1	0.1g	Research - mycelium growing on a liquid culture medium with the addition of crushed wood impregnated with creosote oil
B-2	0.25g	
B-3	0.5g	

The activity of laccase was measured daily from the first to the tenth day from the moment of adding wood samples containing creosote oil to the substrate. Lacase activity was measured using the spectrophotometric method developed by Leonowicz and Grzywnowicz (1981). The substrate used for the enzymatic reaction was syrngaldazine. The increase in absorbance was measured during 60s at the wavelength λ = 525nm. The enzyme activity is expressed by the following formula:

$$As = \frac{\Delta A * Vc * 10^9}{E * dt * Vp}$$

ΔA-przyrost absorbancji
E-molowy współczynnik absorbancji dla syryngaldazyny 65000
dt-60 sek.
Vc-całkowita objętość mieszaniny (1ml)
Vp-objętość próbki (0.1ml)
As-aktywność niespecyficzna (nkat/l)

Before performing enzymatic tests, the amount of impregnating oil in samples of crushed wood was determined. For this, 10 g of wood was added to 200 g of toluene and extracted in an extraction apparatus. The extracted sample was dried and weighed to the nearest 0.01 g. The content of creosote oil in the wood was calculated according to the formula (Lutomski 2002):

$$K = (m_1 - m_2 - m_3)/m_3$$

where:
K- oil content in the sample (g)
m_1- sample weight before extraction (g)
m_2 – water content in the wood sample (calculated from the water difference in the condensate collector before and after the extraction process) (g)
m_3 - dry sample mass after extraction (g)

The retention of creosote oil in wood (kg/m^3) was calculated according to the formula:

$$R = K \times \delta \times 1.2$$

where:
δ – wood density (kg/m^3)
1.2 – correlation coefficient

The results were statistically analyzed by analyzing the variance for a single classification using Snedecor's statistics (F statistics). Statistical inference was performed for the significance level α = 0.05. The means of the individual sample groups were then compared using a multiple comparison test (post hoc test). For this purpose, the Tukey test was used. Statistical analyzes were performed using Statistica version 13.3.

RESULTS

The content of creosote oil in individual weights of wood is presented in Table 2.

Table 2. The content of creosote oil in individual quantities of wood

Sample code	Weight of impregnated wood	Creosote oil content
B-1	0.1g	0.03g
B-2	0.25g	0.08g
B-3	0.5g	0.15g

Based on the data contained in Table 3-5, it was indicated that the studied species of fungi causing white decomposition of wood were characterized by a different activity of the laccase enzyme. *T. versicolor* is a fungus that is highly lactic acid. Laccase activity measured on the tenth day of the *T. versicolor* culture was over 11 times greater than the activity of this enzyme measured in A. borealis culture and 8 times greater than in *P. ostreatus* culture.

Table 3. Activity of laccase in fungi cultivation with the addition of wood impregnated with creosote oil

Fungi	Sample code	Day								
		1	3	4	5	6	7	8	9	10
		nkat/l								
Trametes versicolor	B-1	1883	3698	4303	5455	6880	7285	6698	5867	8466
	B-2	1099	1880	3266	4878	7055	8547	8766	8790	11123
	B-3	1001	2836	3364	4324	8904	9330	9023	8303	13848
Pleurotus ostreatus	B-1	765	628	718	732	720	742	700	585	147
	B-2	173	324	348	356	393	365	374	377	97
	B-3	26	54	50	58	57	83	39	35	8
Armilaria borealis	B-1	5	31	48	40	35	28	26	30	16
	B-2	7	32	50	39	18	15	19	12	6
	B-3	7	17	11	16	7	7	9	7	3

Creosote oil contained in saturated wood and the addition of pure wood influences the activity of the enzyme laccase synthesized by test fungi. Based on the data presented in Table 3, it can be seen that the higher concentrations of the biocide contained in the wood stimulate the fungus *T. versicolor* to synthesize the enzyme with greater activity. It should be noted, however, that at the highest concentration of creosote oil, initially the activity of laccase weakens, however, on the sixth day of cultivation, the enzymatic activity significantly exceeds

the activity of the enzyme compared to trials with a lower proportion of creosote oil. On the tenth day of the B-3 culture, we observe a 60% increase in laccase activity, compared to the sample with the lowest content of creosote oil.

A different pattern of laccase activity was observed in the cultivation of *P. ostreatus* and *A. borealis* fungi. In the case of these fungi, a decrease in the enzyme activity is noticeable with an increasing proportion of impregnated wood in the culture medium. Therefore, it should be concluded that the high biocide content inhibits the growth of *P. ostreatus* and *A. borealis*. The negative effects of the biocide contained in wood are confirmed by the data in Table 4, which illustrate the activity of laccase in the presence of biocide-free wood in the culture fluid. On the basis of the obtained data, it can be concluded that the presence of biocide-free wood stimulates *P. ostreatus* and *A. borealis* cells to synthesize laccase.

At the same time, it should be stated that the activity of the enzyme synthesized by *T. versicolor* is high, but definitely lower than in the biocide samples, which clearly indicates that the components of creosote oil contained in saturated wood stimulate the fungal metabolism to synthesize laccase induced.

Table 4. Activity of laccase in fungi cultivation with the addition of non-impregnated wood

Fungi	Sample code	Day								
		1	3	4	5	6	7	8	9	10
		nkat/l								
Trametes versicolor	KZ-1	643	1686	2255	2556	2691	2476	2286	2349	2219
	KZ-2	815	1815	2440	2795	3198	3155	3043	3071	2837
	KZ-3	1047	2209	3228	3879	4828	4763	4205	4235	4042
Pleurotus ostreatus	KZ-1	59	132	124	150	160	226	337	398	369
	KZ-2	102	365	473	551	618	622	714	772	232
	KZ-3	147	402	594	663	667	753	803	772	281
Armilaria borealis	KZ-1	6	15	17	15	23	36	29	41	54
	KZ-2	8	21	21	14	13	18	38	32	43
	KZ-3	4	9	13	6	6	11	27	27	30

Table 5. Activity of laccase in fungal cultivation on a culture medium (control)

Fungi	Sample code	Day								
		1	3	4	5	6	7	8	9	10
		nkat/l								
Trametes versicolor	KBz	893	855	1109	1281	1476	1512	1537	1626	1794
Pleurotus ostreatus	KBz	43	71	91	140	162	214	223	285	222
Armillaria borealis	KBz	7	14	14	40	53	90	148	140	160

The research showed significant differences in the synthesis of laccase by individual fungi in contact with creosote oil (Table 6).

Table 6. Anova and division of test variants into homogeneous groups for test variants with the addition of wood impregnated with creosote oil

Variant	Average activity of laccase (nkat/l)	$F_{emp.}$	$F_{0.05}$	Tukey's test
T. versicolor -wood+creosote	935.14	363.67	3.03	a
P. ostreatus -wood+creosote	349.76			b
A. borealis -wood+creosote	27.62			c

Table 7 presents the results of all research variants for individual fungi. A statistically significant effect of creosote on the synthesis of laccase by *T. versicolor* was found. In the case of *P. ostreatus*, the addition of wood to the substrate induced the enzymatic activity of the fungus, but no statistically significant differences were found between the activity of laccase synthesized on the substrate with wood saturated with creosote and other research variants. Similar observations were obtained in breeding *A. borealis*.

Table 7. Anova and division of test variants into homogeneous groups for all research variants

Variant	Average activity of laccase (nkat/l)	$F_{emp.}$	$F_{0.05}$	Tukey's test
T. versicolor -wood+creosote	935.14	21.64	3.03	a
T. versicolor - non treated wood	833.78			b
T. versicolor - control	721.92			c
P. ostreatus -wood+creosote	349.76	28.19	3.04	a
P. ostreatus - non treated wood	429.51			a
P. ostreatus - control	79.24			b
A. borealis -wood+creosote	27.62	8.18	3.03	a
A. borealis - non treated wood	21.78			ab
A. borealis - control	14.57			b

The induced synthesis of laccase in the presence of various chemical compounds called xenobiotics in the growth medium of *T. versicor* was observed in numerous studies (Mayer and Staples, 2002, Okazaki *et al*. 2002, Keum and Li, 2004, Andres and Betlej, 2009). Betlej (2011) showed that in the presence of copper in post-consumer wood, derived from wood preservatives, the enzyme induction was significant and may indicate that it is a response of fungal cells to the attempt to neutralize harmful substances. Also, the chemicals contained in creosote oil stimulate fungal cells to induce the synthesis of redox enzymes, which are assigned the role of neutralizing organic compounds by oxidation and reduction. Zeng *et al.* (2018) showed that the ability of laccase to oxidize benzo[a]pyrene reduces the negative effects of this compound on soil organisms. The ability of laccase to degrade such hydrocarbons as phenanthrene, anthracene, benzo[a]anthracene was studied by Li *et al.* (2010). The authors of the research indicated that laccase synthesized by *T. versicolor*, in the presence of mediators, almost doubles the efficiency of the benzo[a]anthracene degradation process than in the absence of such factors. The results obtained in the present study show that the creosote oil contained in wood modulates the activity of the laccase enzyme. This biocide definitely stimulates the cells of the *T. versicolor* fungus to intensify the enzyme synthesis, which may be a conclusion for the design of further research on the biological attempt to biutilize post-consumer wood containing biocides, such as creosote oil

CONCLUSION

1. The addition of sawdust from wood impregnated with creosote oil to the liquid cultures of white decay fungi *T. versicolor* and *P. ostreatus* increased the activity of laccase.
2. The highest activity of laccase in the presence of fragmented wood impregnated with creosote oil was shown by *T. vercicolor*.
3. Laccase activity in the culture of *P. ostreatus* with the addition of crushed wood impregnated with creosote oil was over twenty times lower than in the corresponding cultures of *T. versicolor*.
4. In the case of *A. borealis*, no increase in laccase activity was observed in liquid cultures with the addition of crushed wood impregnated with creosote oil.

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Streszczenie: W artykule przedstawiono wyniki oceny aktywności lakazy, synetyzowanej przez grzyby powodujące biały rozkład drewna w obecności w środowisku wzrostu drewna impregnowanego olejem kreozotowym. Uzyskane wyniki wskazują, że olej kreozotowy zawarty w drewnie działa modulująco na aktywność tego enzymu. Zdecydowanie olej kreozotowy pobudza komórki grzyba *T. versicolor* do indukcji syntezy lakazy, co może mieć duże znaczenie praktyczne z punktu możliwości projektowania biotechnologicznego sposobu biodegradacji zanieczyszczeń, w tym zanieczyszczeń w postaci odpadów drewna impregnowanego.

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