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TOXICITY OF CHLORINATED PHENOLS AGAINST LIGNINOLYTIC FUNGI *HYPOXYLON FRAGIFORME* AND *CONIOPHORA PUTEANA*

Toksičnost hloriranih fenola za lignikolne vrste gljiva *Hypoxylon fragiforme i Coniophora puteana*

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Abstract

Disposal and incineration or recycling of old and waste wood represents an environmental problem. In most cases, agents on the basis of chlorinated phenols were used for wood preservation. These substances penetrate deeper into the wood and because of their chemical stability it remain for decades. This is an environmental problem since chlorinated phenols are among the most acute and chronic toxic contaminants. One of the promising methods to resolve this problem is biodegradation of chlorinated phenols by microorganisms and by fungi, lately. The toxicity of chlorinated phenols against ligninolytic fungi *Hypoxylon fragiforme (Hf)* and *Coniophora puteana (Cp)* has been investigated in this paper. Results showed that the maximum concentration of chlorinated phenols, that allows the growth of *Hp* and *Cp* fungi, is 2.5 mmol/L. Testing the impact of eight chlorinated phenols on the growth of fungi, it was determined that chlorinated phenols show less antifungal activity to *Hp* fungus. Antifungal activity to both fungi increases with the number of substituted chlorine atoms. Also, an important role in antifungal activity, have a physico-chemical properties of chlorinated phenols, primarily LogKow, pKa and the Henry constant. Position of chlorine atoms in the molecule also has a certain influence.

Key words: Chlorinated phenols, fungi, *Hypoxylon fragiforme, Coniophora puteana*

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INTRODUCTION – *Uvod*

Chlorinated phenols (CPs) comprise group of 19 isomers with substituted chlorine atoms. Chlorinated phenols, with less of three substituted chlorine atoms, generally are not widely in use, except as a base compounds for production of others CPs (CPs with more than three chlorine atoms, some herbicides etc.). Pentachlorophenol and some tetrachlophenols have been widely used as a fungicides and agents for wood protection. Contamination of the environment by these chemicals comes from industrial and agricultural waste, during process of removing odors from the water and adding chlorine etc. Acute toxicity of CPs is relatively low, and their chronic effects are not well known. Direct mutagenic, teratogenic and carcinogenic effects of CPs are not proven, but these compounds could act as a promotional and co-carcinogenic substance. Industrial production of CPs comes from hydrolysis of chlorinated-benzenes or chlorination of phenols. Also CPs could be obtained as a semi-products during processes of 2,3-dichlorophenoxyacetitc acid production (KENT AND JAMES, 1983) or whitening of wood pulp (KRINGSTAD AND LINDSTRÖM, 1984). Only seven of chlorophenols (monochlorophenol, 2,4-dichlorophenol, 2,4,6-trichlorophenol, 2,4,5-trichlorophenol, 2,3,4,5-tetrachlorophenol, 2,3,4,6-tetrachlorophenol and pentachlorophenol) have been used in industry. Pentachlorophenol and tetrachlorophenols have been used as plant protecting chemicals, and the mixtures of CPs were used as wood and leather impregnants (KRINGSTAD AND LINDSTRÖM, 1984). Environment contamination by CPs is a serious ecological problem in countries in which had been allowed using agents that contains CPs. These agents mainly are used for conservation of wood and plant protection. Researches of environment pollution are confirmed existence of CPs in many ecosystems: underground and surface waters, sediments, soil and air (SINKKONEN AND PAASIVIRATA, 2000). Under certain conditions, CPs could become substrate for creation of PCBs and dioxin (VOLLMUTH ET AL., 1994; CONNELL, 1997; TUPPURAINEN ET AL., 2000). CONNELL (1997) has established that burning the wood mass, which previously been impregnated with pentachlorophenol, produces emission of dibenzodioxins and dibenzofurans. International organization for cancer research (IARC) has published, in 1987, information about appearance of cancer in people who work in storages of wood material and saw-mills. These types of cancers primarily include: nose, lymph, leukemia and sarcoma of soft tissue (IARC, 1987; HUFF, 2001).

Lignin is, beside cellulose and hemicelluloses, the main component of wood material and most abundant form of aromatic carbon in the biosphere. Type of chemical bonds in lignin and their heterogeneity, do not allows usually degradation by hydrolytic enzymes, process that is present in the others natural polymers (cellulose, starch, proteins etc.). Decomposition of lignin is the oxidation process, which fungi arouse using their extracellular enzymes (HATAKKA, 1994; LUNDSTEDT, 2003). Ability of fungi to transform different hazard chemicals has initiated interests for their usage in bioremediation (ALEXANDER, 1994). Many researches are shown that white-rot fungi

are able to degrade various aromatic pollutants (KOTTERMANN ET AL., 1994; BAZEL ET AL., 1996; COLLINS ET AL., 1996).

This work investigates the influence (toxicity, growing etc.) of CPs to white rot fungus (*Hypoxilon fragiforme*) and brown rot fungus (*Coniophora puteana*), and the reason for testing is fact, that could not be found enough data about this relation in the relevant databases. White rot and soft rot fungi are unique among eukaryote because have specific methods for lignin degradation but do not use lignin as a source of food (KIRK ET AL., 1976). Brown rot fungi, as a nutrient, uses cellulose and hemicellulose from cell wall of plants, at same time lignin stay non-decomposed (MICALES AND HIGHLEY, 1988). On the other side, brown rot fungi are able to modify lignin, and that is reported by JIN ET AL., (1990).

Aim of the present work is: estimate maximum concentration of CPs that is not toxic for tested ligninolytic fungi (*Cp* and *Hf*); make evaluates of antifungal activity of selected CPs set, against white rot (*Hf*) and brown rot (*Cp*) fungi; find the correlation of number and position of substituted chlorine atoms in the CPs and physical-chemical properties of CPs, according their antifungal activity.

MATERIALS AND METHODS - *Materijali i metode*

Ligninolytic fungi -*Lignikolne gljive*

Both of the cultures *Hypoxylon fragiforme* (Pers.: Fr.) J. Kickx fil., ZIM L 108 KPZL 508, 1990, orig. mark CBS *Hf* and *Coniophora puteana* (Schumacher ex Fries) Karsten ZIM L 010, 1995, orig. mark FCo58 *C,* belong to the collection of the Department of Wood Science and Technology, Biotechnical Faculty, University of Ljubljana, Slovenia. (RASPOR ET AL., 1995).

ZIM – Zbirka industrijskih mikroorganizama. (The collection of industrial microorganisms).

Chlorinated phenols - *Hlorirani fenoli*

3,4-dichlorophenol (3,4-DCP); 3,5-dichlorophenol (3,5-DCP); 2,3,6-trichlorophenol (2,3,6-TCP); 2,4,5-trichlorophenol (2,4,5-TCP); 2,4,6-trichlorophenol (2,4,6-TCP); 2,3,4,6-tetrachlorophenol (2,3,4,6-TeCP); 2,3,5,6-tetrachlorophenol (2,3,5,6-TeCP) and Pentachlorophenol (PCP). Tested CPs are 98-99,9 % purity, produced by Aldrich and SUPELCO.

Table 1. Some physical and chemical properties of the tested chlorinated phenols
Tabela 1. Neke fizičko-hemijske osobine testiranih hloriranih fenola

Serial number	Compound (Abbreviation)	Log K_{ow} [a]	Solubility in water (g/L) (20 ° C)[a]	Henry constant Pa m^3/mol, 25 °C)[a]	pK_a [a]
1.	3,4-DCP	3,33	< 0,01	0,03	8,63
2.	3,5-DCP	3,62	< 0,01	0,03	8,18
3.	2,3,6-TCP	3,77	< 0,01	0,023	5,80
4.	2,4,5-TCP	3,72	0,948	0,16	7,40
5.	2,4,6-TCP	3,69	0,434	0,26	6,23
6.	2,3,4,6-TeCP	4,45	0,183	0,15	5,22
7.	2,3,5,6-TeCP	4,90	0,100	0,035	5,14
8.	PCP	5,12	0,014	0,0025	4,70

[a] HSDB - Hazardous Substances Data Bank; Czaplicka (2004)

Determination of maximum CPs concentration that is not toxic for ligninolytic fungi – *Određivanje maksimalne koncentracije CP - a koja nije toksična za lignikolne gljive*

Before beginning the analysis, it were performed monitoring tests on a solid medium, for the determination of maximum non-hazardous concentrations of CPs. Monitoring test included nine different concentrations of working solutions of each of the selected CPs ranging from 20 to 0.078 mmol/L (20/10/5/2,5/1,25/0,625/0,313/0, 156/0,078 mmol/L). Solutions of tested CPs (Table 1) were prepared in DMSO (Fluka, 99,9 %). Cultures of fungi were grown on a 4 % (w/v) potato dextrose agar medium (PDA, DifcoTM, BECTON DICKSON, USA) which previously being sterilized by autoclaving at 121 ºC, 20 min. Petri dishes (Ø 90 mm, plastic, Golias labortehnika) with PDA medium were inoculated with small pieces (Ø 5 mm) of *Cp* and *Hf* fungal mycelium, in the Laminar flow (TELASTAR AH -100, JosepTapiolas 120, 08226 TERRASSA, Spain).The hyphae mycelium for inoculation were taken from the same fungus (laboratory culture) that had previously grown on a PDA base under the same conditions such as the above mentioned but without the presence of any other substances. At same time, on the edge of Petri dishes, around micelles, have been set five Whatman papers (AA DISCS 13 mm). One paper was used as a control test and it did not add anything, on the next paper was added 0,1 mL DMSO. On the next three was added per 0,1 mL of solution, which contained CPs in DMSO solvent, in the following concentration: 20/10/5 in the first; 2,5/1, 25/0,625 in second; and 0,313/0,156/0,078 mmol/L in third Petri dish (Figure 1).

Each experiment was carried out in three replicates. Petri dishes were sealed using transparent foil and placed in the dark chamber for growing (Combi Cold Rac Laboratory Refrigirator 5201). Conditions in the chamber were the follows: temperature 25 ^{o}C and relative humidity 75 %.

Impact of selected CPs set, fixed concentration, to the growing of ligninolytic fungi – *Uticaj odabranog seta CP-a fiksne koncentracije na rast lignikolnih gljiva*

On the sterilized PDA media, in the center of Petri dishes, has been inoculated one micelle from one fungus. Around, were set three Whatman papers (WP), and on the one of them was added 0,1 mL of selected solution (CPs in DMSO, c = 2,5 mmol/L). Another paper was been moist with 0,1 mL DMSO, and third remained dry (control paper) (Figure 2). All tests (CPs in DMSO, only DMSO and the control paper) were conducted in three replicates. Replicates prepared on this way, were closed with transparent foil, placed in darken incubator, and left to growing at temperature of 25 º C and relative humidity of 75 %.

RESULTS - *Rezultati*

Growth of *Hf* fungus was monitored for seven days, while the growth of *Cp* fungus was monitored for twenty days. Expansion (growth) of the mycelium was measured in millimeters, from the center of Petri dish, i.e. from the middle of the inoculated mycelium to its edge and towards the Whatman papers (Figure 1). One example of antifungal activity of 2,4,6-trichlorophenol could be seen on the Figure 1. Both fungi show a similar behavior but it is evident that *Cp* is growing slowly than *Hf*. After a certain period, the growth of fungus was measured under control, DMSO and substances with the appropriate concentration. The results were taken as an average value of three parallel samples. From the results of monitoring tests it is clear that higher concentration of CPs (20 to 5 mmol/L) shows a stronger antifungal activity compared to lower concentration CPs, that is they completely prevent the growth of fungus *Cp* while the fungus *Hf* grew up to 8 mm in seven days with the presence of tetrachlorophenol, but there was no growth with the presence of pentachlorophenol. A lower set of concentrations CP_s 0,313/0,156/0,078 mmol/L almost does not prevent the growth of fungi, so that the fungus *Hf* for a period shorter than seven days and fungus *Cp* for a period shorter than twenty days have grown to the edge of Petri dish. The monitoring of the growth of fungi was best at an average set of concentrations 2,5/1,25/0,625 mmol/L, when the fungus *Hf* has grown in 20 days on average, from 45 mm with dichlorophenol to 30 mm with pentachlorophenol; and fungus *Cp* has grown in 20 days from 35 mm with dichlorophenol to 25 mm with pentachlorophenol.

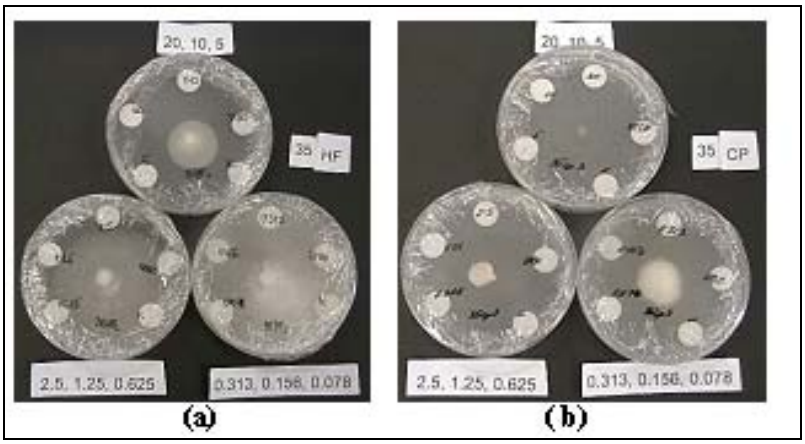

Figure 1. The growth of fungi *Hf* **(a)** and *Cp* **(b)** in the presence of various concentrations of 2,4,6-trichlorphenol

Slika 1. Rast gljive Hf ***(a)*** *i gljive Cp* ***(b)*** *uz prisustvo različitih koncentracija 2,4,6-trihlorfenola*

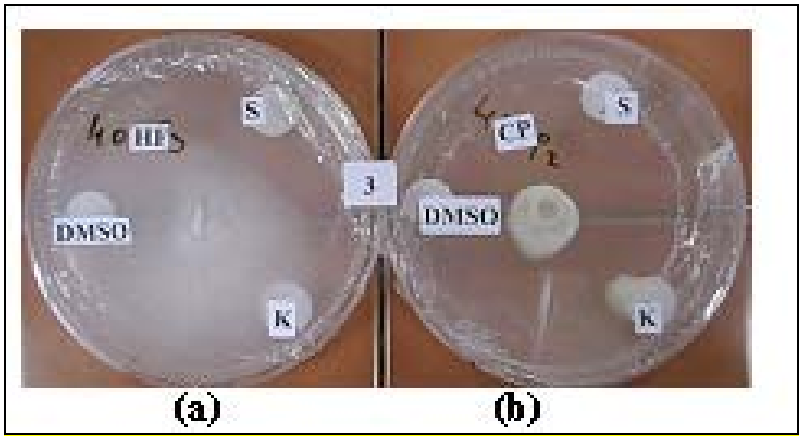

Figure 2. The growth of fungi *Hf* **(a)** and *Cp* **(b)**, on the PDA-gel in the presence of control Whatman papers (K), pieces of paper with DMSO and the substance (S) (pentachlorophenol) three days after incubation

Slika 2. Rast gljive Hf ***(a)*** *i gljive Cp* ***(b),*** *na PDA-gelu u prisustvu kontrolnog Whatman papirića (K), papirića sa DMSO i sa supstancom (S) (pentahlorfenol) tri dana nakon inkubacije*

According to the obtained results it is concluded that the concentrations of CPs, which allows growing of fungi in measurable quantities, are 2,5 mmol/L and lower. As an optimal concentration the value of 2,5 mmol/L has been chosen. After certain period it was measured a size of fungi (mm), from the center of micelle to edge of Petri dish. Dimension of fungi were noted after 3, 7, 10 and 20 days from inoculation. *Cp* fungus grows slowly than *Hf* and achieves maximum after 20 days, *Hf*

has a maximum size on the 7th day of growing. The appearance of fungus mycelium in the presence of pentachlorophenol (concentration of 2.5 mmol / L), three days after inoculation is shown in Figure 2. Results of growing *Hf* and *Cp* fungi, in the presence of tested CPs, are shown on the Figures 3 - 6. *Hf* fungus grows much faster than *Cp*, spatially, but as regards biomass it is not certain. That was a reason for short period of testing of this fungus (only 7 days, instead of planed 20). Growing of *Cp* fungus for 20 days, to the control paper; paper with DMSO; and paper with CPs in DMSO, is shown graphically on the Figure 3.

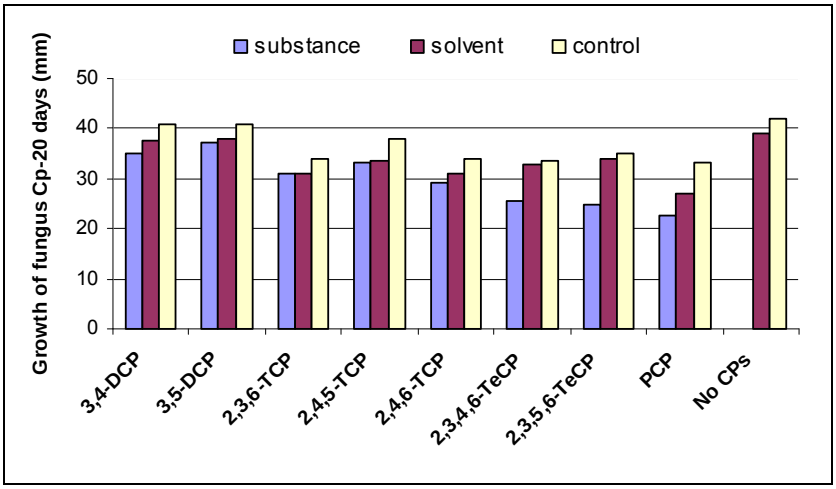

Figure 3. The growth of fungus *Cp* (mm) toward the edge of the container, or Whatman paper with CPs, solvent and control Whatman paper
Slika 3. Rast gljive Cp (mm) prema rubu posude, odnosno Whatman papirićima sa CP-om, otapalom i kontrolnom Whatman papiriću

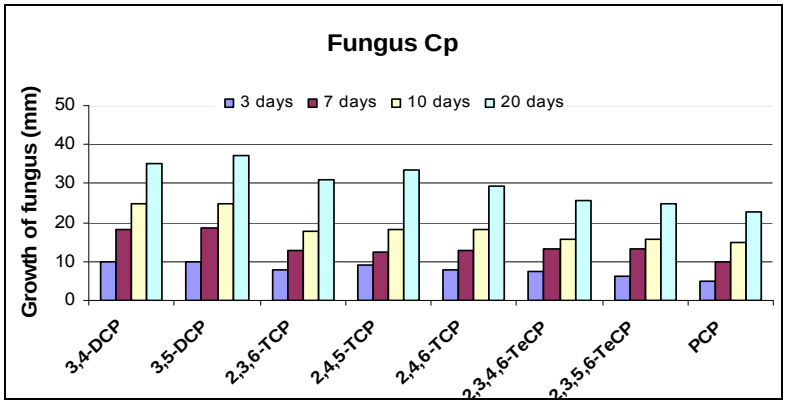

Figure 4. Growth of fungus *Cp* to substances (CPs) for different times
Slika 4. Rast gljive Cp prema supstanci (CP) kroz različito vrijeme

Growing of *Cp* fungus, on paper with CPs, for the period of 3, 7, 10 and 20 days, is shown on the Figure 4.

Growing of *Hf* fungus for 3 and 7 days of incubation, to the control paper; paper with DMSO; and paper with CPs in DMSO, is shown graphically on the Figure 5.

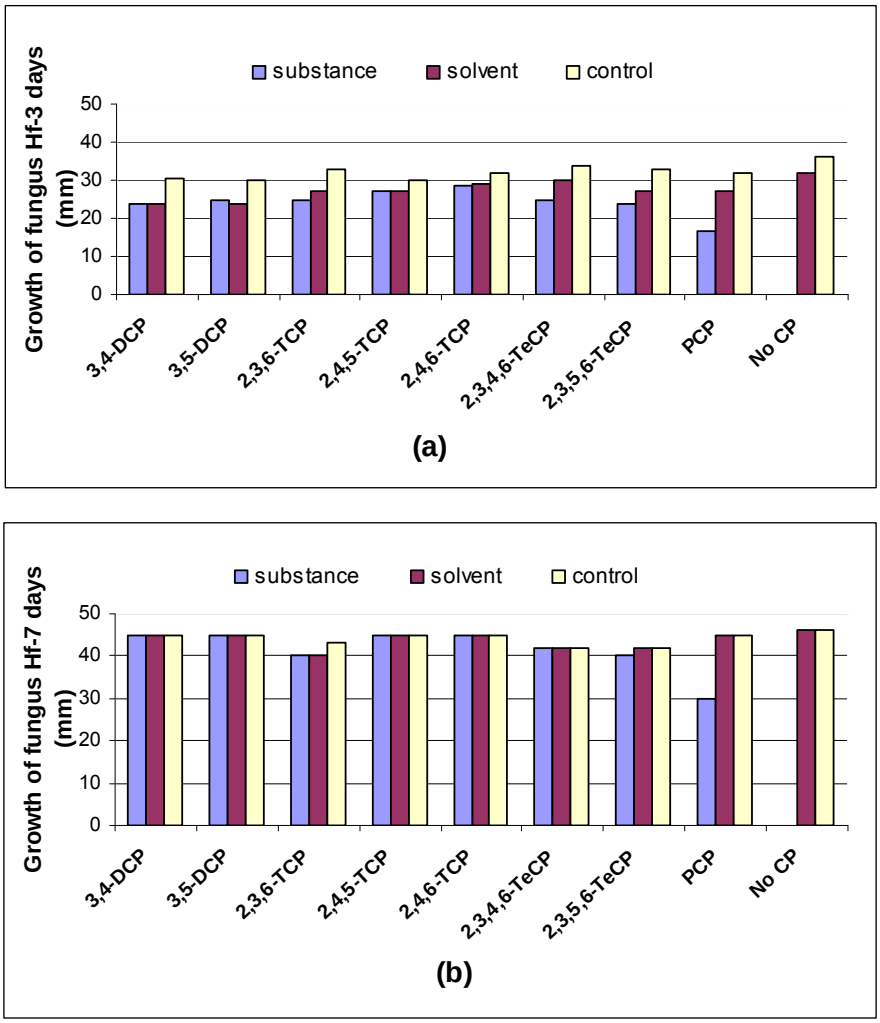

Figure 5. The growth of fungus *Hf* (mm) toward the edge of the container, or Whatman paperwith CPs, solvent and control Whatman paper after 3 days **(a)** and 7 days **(b)**
*Slika 5. Rast gljive Hf (mm) prema rubu posude, odnosno Whatman papirićima sa CP-om, otapalom i kontrolnom Whatman papiriću nakon 3 dana **(a)** i nakon 7 dana **(b)***

Growing of *Hf* fungus, to the paper with CPs for the period of 3 and 7 days, is shown on the Figure 6.

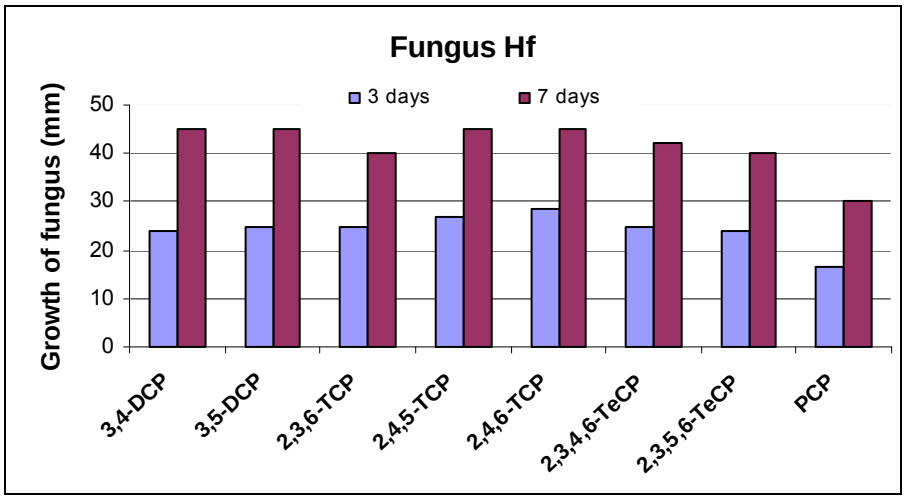

Figure 6. Growth of fungus *Hf* to substances (CPs) for different times
Slika 6. Rast gljive Hf prema supstanci (CP), kroz različito vrijeme

Table 2. Correlation coefficient between the growth of fungi and physical and chemical properties of CP
Tabela 2.Koeficijent korelacije između rasta gljive i fizičko-hemijskih osobina CP

Physical and chemical properties / Fungi	Number of chlorine atoms	Log K_{ow}	Solubility in water	Henry constant	pK_a
Cp	-0,96	-0,91	0,14	-0,01	0,94
Hf	-0,83	-0,82	0,39	0,49	0,72

DISCUSSION – *Diskusija*

Ligninolytic fungi are probably only group of organism who are able to carry out mineralization of lignin completely. Enzymatic system of ligninolytic fungi: lignin peroxidase (LiP); manganese peroxidase (MnP); and laccase (Lac), mainly are non-specific and extracellular and acting oxidative in the process of biodegradation. Activity of ligninolytic enzymes becomes intense in the second phase of metabolism during lack of nutritive substances, especially nitrogen and carbon. In those situation fungi uses the organic compounds for their metabolism, primarily those compounds that are similar to lignin in its structure.

It is known that is ligninolytic chain created by simple parts of p-hydroxycinnamyl alcohols, such as: p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol which are similar to CPs in their structure. Accordingly the white rot fungi are used in degradation of poliaromatic hydrocarbons, phenols and their chlorinated derivates. There is minor number of scientific articles for the fungi tested in this research, particularly relating to *Hf*. Acting of fungi on the CPs arising by the lignin-degradated enzymes, laccase and Mn-peroxidase. CPs are known substrates for the oxidative enzyme - laccase, which has effect to the all CPs including PCP (KADHIM ET AL., 1999; ULLAH ET AL., 2000a). CPs are also substrates for MnP which moreover needs H_2O_2 and Mn (II) ion for reaction (MILSTEN ET AL., 1992).

KADHIM ET AL. (1999), ULLAH ET AL. (2000b) and LEONTIEVSKY ET AL. (2002b) were reported, that in the *Trametes versicolor* fungus, laccase is the main enzyme included in degradation of CPs. Previously, LYR (1963) was reported ability of *Trametes versicolor* to makes de-chlorination of CPs to non specific products. Subsequent research are confirmed this notice and have showed that white rot fungi decompose wide spectra of CPs, and many of them are significant contaminants which resulting from process of wood pulp whitening by chlorine. Monitoring of the fungus growth could be a base for the estimation of maximum concentration of CPs that does not inhibit growing of fungi.

Analyzing the results (Figure 3 and 5) it is evident that growth of *Cp* and *Hf* to the control paper is more than to the paper with substance. It is clear that CPs, in the concentration of 2,5 mmol/L, have an antifungal activity to tested fungi. This activity is stronger with the increasing of number of chlorine atoms in the CPs molecule. More detailed analysis has shown that, in the initial phase, *Hf* grows much faster to the control paper, in all cases (Figure 5). In the later period, fungus grows maximal in all directions (except pentachlorophenol) (Figure 2 and 5b). After certain period *Hf* was adapted on the CPs (except PCP) and it was used as source of food. Analysis of *Cp* and *Hf* growth to the paper with sample, for certain period, indicates linear growth in the function of time (Figure 4 and 6). Also, there is an evident slowing of fungus growth against increasing of substituted chlorine atoms, like is reported by ANNACHHATREA AND GHEEWALA (1996).

Reason for slowly growth of fungus is obviously the toxicity of the CPs with a large number of substituted chlorine atoms. Out of this rule is 2,4,5-trichlorophenol in this case the *Cp* fungus had a faster growth in the later period, opposed to the other two trichlorophenols. Related to *Hf* fungus, a clear is slower growth with tetrachlorophenols and pentachlorophenol than with other two phenols with two and three substituted chlorine atoms. Also, in this case there is an exception, and it is 2,3,6-trichlorophenol which had a greater antifungal activity. Obviously chlorine on the position 3, in the phenol ring, expresses stronger influence on the *Hf* fungus than others two trichlorophenols.Table 2 shows that the growth of fungi is in direct relation with the number of substituted chlorine atoms. In fact, the values of correlation

coefficients between the growth of fungi and the number of chlorinated phenols show a high, that is a very high correlation of these values (PETZ, 2004).

Antifungal activity of CPs could be related with their phys-chemical properties (Table 1 and Table 2). 2,3,6-TCP has a lower solubility in water; lower value of Henry's constant; lower pKa and higher value of logKow, compared to other two tested trichlorophenols. 2,3,6-TCP has a higher toxicity effect to the *Hf*, compared with the others dichlorophenol and trichlorophenols, and could be comparable with toxicity of tetrachlorophenol. Pentachlorophenol limits growth of both fungi, more than the other tested CPs, and its toxicity is much more expressed to the *Cp* than *Hf*. (Figure 2). 2,3,5,6-tetrachlorophenol shows higher antifungal activity than 2,3,4,6-tetrachlorophenol, it means that symmetry of chlorine atoms has some influence on the antifungal activity, precisely more symmetry - stronger antifungal activity. By analyzing the correlation coefficients of the growth of fungi with the physical and chemical properties (Table 2), it is evident that the growth of both fungi according to chlorinated phenols has a high correlation with Log Kow and pKa tested phenols while the solubility in water and Henry constant have a slight correlation with the growth of fungi *Hf* but no correlation with the growth of fungi *Cp*.

This study was shown that CPs, in the concentration of 2,5 mmol/L, have a partly toxicity against tested fungi, because they limits their growth more than alone DMSO solvent. DMSO solvent also shows some level of toxicity to the fungi, because to the paper with DMSO fungi grow slowly than in the control sample.
Regardless to the partly limitation of fungi growing to the paper with CPs in the concentration of 2,5 mmol/L, could be concluded that this concentration does not show highly antifungal activity to the *Hf* and *Cp*.

Comparison of growing for both tested fungi leads to conclusion that CPs are less toxic for the *Hf* than *Cp*. Comparison of antifungal activity of CPs and their phys-chemical properties (Table 1) shows direct proportionality of antifungal activity and LogKow values, i.e. higher LogKow - higher antifungal activity (except two mentioned cases).

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SAŽETAK

Kontaminacija životne sredine hloriranim fenolima dolazi od industrijskih efluenata, otpadaka u poljoprivredi, herbicida hlorfenoksisirćetne kiseline, zatim od heksahlorbenzena, spontanog formiranja hloriranih fenola dezinfekcijom i uklanjanjem mirisa iz vode tokom procesa hloriranja a takođe i odlaganjem otpadnog drveta koje je najčešće zaštićeno pentahlorfenolom i nekim tetrahlorfenolima. Odlaganje i spaljivanje odnosno reciklaža staroga i otpadnog drveta pretstavlja ekološki problem. Jedna od obećavajućih metoda u pogledu rješavanja ovog problema pretstavlja biorazgradnja hloriranih fenola pomoću mikroorganizama a u posljednje vrijeme i pomoću gljiva. U radu je ispitivana toksičnost hloriranih fenola za lignikolne vrste gljiva *Hypoxylon fragiforme* (*Hf*) i *Coniophora puteana* (*Cp*). Za ove gljive nema podataka da su ranije testirane a poznato je da je gljive bijele truleži (*Hf*) imaju razvijene nespecifične metode za degradaciju lignina. Gljive smeđe truleži, pak među koje spada *Cp,* za svoj rast koriste hemicelulozu i celulozu iz ćelijskog zida biljaka, ostavljajući lignin nerazgrađen. Zapaženo je, međutim, da gljive smeđe truleži modificiraju lignin. Lignin, skupa sa celulozom i hemicelulozom, je glavna komponenta drvnog materijala i najobimnija forma aromatskog ugljika u biosferi. Zbog tipova veza i njihove heterogenosti, lignin ne može biti razgrađen hidrolitičkim enzimima kao većina drugih prirodnih polimera (celuloza, škrob, proteini itd). U radu je određivana maksimalna koncentracija osam hloriranih fenola (CP-a) koja nije toksična za odabrane lignikolne gljive. Testirani su 3,4-dihlorfenol; 3,5-dihlorfenol; 2,3,6-trihlorfenol; 2,4,5-trihlorfenol; 2,4,6-trihlorfenol; 2,3,4,6-tetrahlorfenol; 2,3,5,6-tetrahlorfenol i pentahlorfenol. Po 0,1 mL devet otopina CP-a u DMSO različitih koncentracija od 20 do 0,078 mmol/L, dodato je na Whatman papiriće na rubu Petrijeve posudi u kojoj je na sredini inakulirana micelija gljive prečnika 5 mm. Hlorirani fenoli (svaki odvojeno i samo jedne koncentracije), testirani su na toksičnost prema pojedinoj gljivi. Došlo se do rezultata da je maksimalna koncentracija hloriranih fenola koja dopušta rast *Hf* i *Cp* gljiva 2,5 mmol/L. U daljem radu praćen je uticaj odabranog seta CP-a koncentracije 2,5 mmol/L.na rast lignikolnih gljiva *Hypoxylon fragiforme* i *Coniophora puteana*, tako što je na sterilnoj podlozi, krompirovog dekstrozo agara u Petrijevim posudama inokulirana na sredini po jedna micelija odgovarajuće gljive. Okolo su postavljena tri Whatman papirića pri čemu je na jedan dodano 0,1 mL testirane supstance otopljene u DMSO, (2,5 mmol/L), na drugom 0,1 mL čistog otapala DMSO a na trećem nije bilo supstance (kontrolni papirić). Testovi su provedeni sa tri jednake paralelke. Rezultati testa su pokazali da manju antifungalnu aktivnost hlorirani fenoli pokazuju prema gljivi bijele truleži (*Hf*) nego prema gljivi smeđe truleži *(Cp).* Antifungalna aktivnost CP-a za obje gljive raste sa povećanjem broja supstituiranih atoma hlora. Raspored atoma hlora u molekuli takođe ima određeni uticaj. Takođe, važnu ulogu u antifungalnoj aktivnosti imaju fizičko-hemijske osobine hloriranih fenola, prije svega LogKow, pKa i Henrijeva konstanta i topivost u vodi. Određeno je da CP-i manje topivi u vodu, koji imaju manju vrijednost Henrijeve konstante, nižu vrijednost pKa i višu vrijednost za logKow više su toksičani za gljivu *Hf* .