Biodegradation of Wood Treated with Copper Based Preservative by Two Dematiaceous Fungi: *Alternaria Tenuissima* and *Ulocladium Consortiale*

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As cases of treated wood colonization by fungi still happen, the understanding of fungal survival abilities could contribute in the creation of new efficient preservatives. For that reason, two dematiaceous fungi *Alternaria tenuissima* and *Ulocladium consortiale* isolated from treated wooden joists with discolorations were tested for their wood decomposition ability and tolerance to a copper based preservative. Our results indicated that the copper based preservative failed to suppress completely cellulose and lignin biodegradation by discoloring fungi studied in wood. Moreover, cellulose degradation was higher in the treated sawdust for both fungi after 30 days than in the untreated. Comparing the results of two strains *U. consortiale* that is little studied as a wood decomposer, was stronger lignin degrader than *A. tenuissima* in treated and untreated sawdust. The copper sulphate tolerance test showed that both fungi were able to grow up to 7 mM concentration in a solid medium.

Keywords: treated wood, biodegradation, lignocellulolytic enzymes, soft rot fungi.

1. INTRODUCTION

In spite of the great assortment of wood preservatives the problem of wood colonization by mold, wood rot and blue stain fungi still exists in both outdoors and indoors. These fungi may cause substantial losses in wood properties, decrease the esthetic value of the wood and even poses a menace to human health [1, 2].

Mold, blue stain and soft rot fungi belong to the group *Ascomycetes* and its related asexual stages [1, 3]. A strict delineation between mold, blue stain and wood decay is not possible since many fungi traditionally implicated in the discolouration of wood can cause soft rot if suitable conditions are prevailing (e.g. *Alternaria alternata, Cladosporium herbarum, Aspergillus fumigatus*) [3, 4].

Soft rot is considered to be the first decay type found in a general sequence of wood colonization [1, 3]. The decay pattern caused by these fungi is either through the development of characteristic cavities aligned with cellulose microfibrils within secondary walls (Type I) or through erosion of cell walls from the lumen (Type II) [4, 5]. These fungi are widespread in nature causing deterioration of wood in aquatic and terrestrial environments [6]. Furthermore, they are particularly active where brown- and white-rot fungi are inhibited by factors such as high moisture content, low aeration, high temperatures and presence of preservatives [1, 7, 8].

Copper-based wood preservatives have been among the main wood preservatives used to protect wood from fungal decay [9, 10]. Copper as well as other heavy metals, when present in excess, can inhibit the growth, cause morphological and physiological changes and affect the reproduction of fungi. In contrast with other essential metals, copper is toxic to most fungi even at very low concentrations. However, soft rot fungi show high copper tolerance with some species/strains growing on 10 % (w/v) copper sulphate incorporated into agar or solid media [11, 12].

Since soft rot appears to predominate in substrates that do not favor the growth and development of other types of fungi, it is necessary to develop a better database on the diversity of soft rot fungi as well as to investigate their physiological-biological properties on treated wood.

Numerous black discolorations were observed on the joists of wooden building though the anti-rot wood preservative has been used for the protection. Two dematiaceous fungal strains were isolated from these patches. In order to find out the reason of treated wood discoloration the aim of the present study was to identify these fungal agents and to evaluate their tolerance to copper and their ability to degrade lignocellulose in the treated sawdust.

2. MATERIALS AND METHODS

Two fungal isolates obtained from treated pine joists, where wood discoloration was observed, were used in this study. The isolates were maintained on PDA (Carl Roth GmbH+Co.KG) slants stored at 4°C, and deposited in the Culture Collection of the Laboratory of Biodeterioration Research in Nature Research Centre (Lithuania).

For molecular identification fungal isolates were cultured on Potato Dextrose Agar (PDA) plates at 26 °C for 7 days. Genomic DNA was extracted using ZR Fungal/Bacterial DNA MiniPrep kit (Zymo Research, USA) according to manufacturer's protocol. The internal

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transcribed spacer (ITS) regions were amplified by PCR using the primers ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') and ITS5 (5' GGA AGT AAA AGT CGT AAC AAG G-3') [13]. Purified PCR products were sequenced in both forward and reverse directions by commercial company (BaseClear, Leiden, the Netherlands). Raw sequence data were analyzed using Bioedit program Version 7.1.9 [14] and were compared with those in NCBI's BLAST (Basic Local Alignment Search Tool) database in order to assess the similarity with published sequences. In order to confirm the molecular identification, a macroscopic and microscopic analysis of taxonomic traits was also performed [15 – 18].

Copper tolerance was estimated by growing fungi on PDA amended with copper sulphate of different concentrations. The concentration of a prepared stock solution of copper sulphate ($CuSO_4$ x $5H_2O$) was equal to 0.25 M. Copper sulphate solution was aseptically added in flasks with sterile molten PDA to achieve the following final concentrations in the medium: 0.5, 1, 2, 3, 5, 7 and 9 mM. The obtained media then were poured into 90 mm diameter Petri dishes. Copper sulphate-free PDA plates served as a control. The agar surface was inoculated with fungi by a needle in the center of the dish and incubated at 26 °C for 7 days. Three replicates were used for each fungus. After incubation, colonies of the fungi were measured perpendicularly and means were calculated. The relative colony diameter (*D*) on the plates with copper sulphate for each fungus was calculated according to the formula:

$$D = b/a \times 100\ \%, \quad (1)$$

where a – mean value of the fungal colony diameter (mm) in plates without copper (control), b – mean value of fungal colony diameter (mm) with copper.

Fungal ability to degrade wood (lignocellulose) was evaluated by studying their enzymatic activity. Phenoloxidases (peroxidase, laccase, tyrosinase) and endoglucanase activities of the fungi were estimated under solid-state fermentation conditions using treated sawdust of Scots pine (*Pinus sylvestris*). The sawdust of pine was passed through 2 mm mesh sieve and soaked with the copper-based anti-rot wood preservative "Erlitas" for 24 hours. The treated sawdust was seasoned for a period of 2 weeks to allow complete fixation. Erlenmeyer flasks (100 ml) were filled with 1 g of the treated sawdust and sterilized by overheated steam. After sterilization, the sawdust was soaked with 3 ml per flask of the nutrient solution (NH_4NO_3 3 % and KH_2PO_4 1 %). The untreated sawdust was used as a control. Then each flask was then inoculated with two 9-mm discs of fungal cultures. Three sets of triplicate flasks were inoculated and after 30 and 90 day incubation at 26 °C temperature the content of cellulose and lignin was evaluated in the treated and untreated sawdust.

The content of cellulose was evaluated following the method of Kürschner's and Hafer's [19]. 1 g of sawdust material was placed in 300 ml flasks containing 30 ml mixture of HNO_3 and C_2H_5OH (in ratio 1:4) and boiled for 1 h. Then the content was filtered and washed with alcohol. The sawdust was then dried and transferred into flasks containing 50 ml 0.3 M NaOH and boiled for 1 h after fitting with a return condenser. After filtration, the filtrate was washed with H_2O. The filter with cellulose was dried at 105 °C for 4 h and weighed.

The content of lignin was evaluated following the method of Chudiakova [20]. For the estimation of lignin degradation 1 g of sawdust material was added to 60 ml of 2 % HCl and boiled for 2 h. The residual sawdust material was then dried and transferred into 7 ml of 72 % H_2SO_4. Hydrolysis was allowed to proceed for 2.5 h. Water (93 ml) was then added to each flask and the contents boiled for 1 h after fitting with a return condenser. The filter plus lignin was then dried at 105 °C for 4 h and weighed. The relative content loss (C) of cellulose and lignin was calculated according to the formula: $C = c_o - c_x/c_o \times 100\ \%$, where c_o – initial content, c_x – content after the experiment.

Endoglucanase (3.2.1.4) activity was estimated with Na-carboximetilcellulose (Na-CMC). 1 ml of 1 % Na-CMC was placed in a test-tube containing 1 ml of a cultural filtrate and incubated at a temperature of 40 °C for 30 min [21]. After incubation endoglucanase activity was estimated according to the amount of the reducible substance in 1 ml of the reaction compound using o-toluidine reagent. The reaction mixture containing 1 ml of enzyme extract and 7 ml of o-toluidine reagent was boiled for 10 min. The amount of glucose was measured spectrophotometrically (Evolution 60S, ThermoFisher Scientific). The absorbance was read at 560 nm. The amount of the reducible substance was estimated according to the glucose calibration graph. Endoglucanase activity was expressed as units (U) g^{-1}.

The assay method for peroxidase (E.C. 1.11.1.7) activity is based on the colorimetric evaluation of the oxidation product of o-dianisidine in the presence of H_2O_2 [21]. Activity was calculated according to the coefficient of micromolar extinction, which is 0.0128. Peroxidase activity was expressed as activity units (AU) g^{-1}.

Tyrosinase (EC 1.10.3.1) activity was measured spectrophotometrically using a method based on the estimation of optical density of reaction products formed during oxidation of pyrocatechin over a given period [19]. Enzyme activity was expressed as conditional units (CU) g^{-1}.

Laccase (E.C. 1.10.3.2) activity was measured according to Ravin and Harward [22]. The reaction mixture contained 0.1 ml enzyme extract, 1 ml 0.5 % p-phenylenediaminechloride and 2 ml 0.1 N acetate buffer (pH 6). The reaction was stopped by adding 1 ml 0.1 % sodium azide solution. The absorbance was read at 530 nm. Laccase activity was expressed as units (U) g^{-1}.

The software package Microsoft Excel version 14.0 was used for data processing and for graphical presentation of the results.

3. RESULTS AND DISCUSSION

3.1. Identification of fungi

According to the molecular and morphological analysis, two isolates from treated wood were identified as *Ulocladium consortiale* (100 %) and *Alternaria tenuissima* (100 %) (Fig. 1). The sequences determined in this study have been submitted to GenBank under accession numbers MF061312 and MF061313. Among the genus *Alternaria*, Lee et al. [23] identified *A. tenuissima* as a dominant species isolated from treated wood samples.

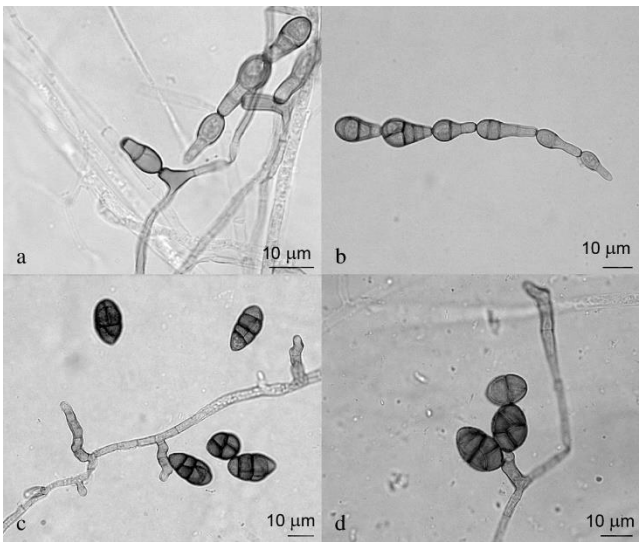

Fig. 1. a, b – *Alternaria tenuissima*; c, d – *Ulocladium consortiale* conidia and conidiophores

In addition, several researchers also indicate A. *tenuissima* or *A. alternata* as the only species of this genus isolated from wood or wood products [24, 25]. Both *A. tenuissima* and *A. alternata* are involved in wood discoloration [23].

In contrary, probably because of the complicated identification of *Ulocladium* and other related genera, there is little published data on *U. consortiale* inhabiting woods [15]. Nilsson [26] determined that *U. consortiale* causes soft rot (type II). However, *U. consortiale* is more commonly known as a plant pathogen infecting tomatoes and cucurbits, also causing diseases in caraway seedlings and olive plants [27].

3.2. Copper sulphate tolerance of *A. tenuissima* and *U. consortiale*

The experiments on tolerance towards copper showed that both fungi rather similarly tolerated copper sulphate, although *A. tenuissima* showed slightly better growth on copper sulphate-amended plates than *U. consortiale* (Fig. 2, Fig. 3). The most striking results were that *U. consortiale* at the lowest (0.5 mM) copper sulphate concentration even exceeded the growth on the control plate.

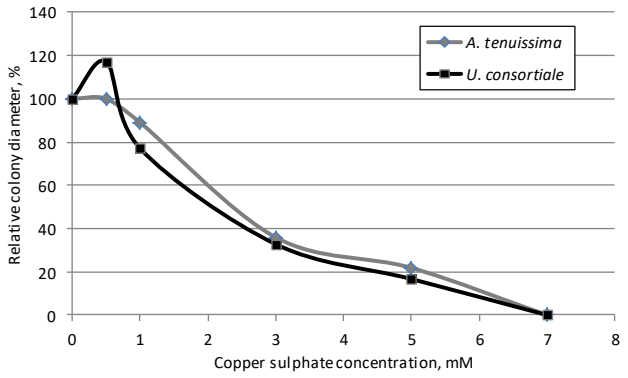

Fig. 2. Relative colony diameter (%) of *Alternaria tenuissima* and *Ulocladium consortiale* colonies on PDA medium ammended with different copper sulphate concentrations

Both fungi were able to grow up to 7 mM concentration in a solid medium and no growth was noticed at higher concentrations of the salt. No changes of conidium production of both fungi were observed with increasing copper concentration. Overall, the results demonstrated that these dematiaceous fungi tolerated quite high concentrations of copper sulphate when compared with other fungi. For example, Shoaib et al. [28] reported that *Alternaria alternata* was able to grow at 90 ppm of $CuSO_4$. The production of melanin by the dematiaceous fungi is considered to be the main mechanism of their tolerance to heavy metals, as it can act in the extracellular precipitation in the adsorption of heavy metals, preventing their action on fungi [29]. Guillén and Machuca [30] showed that wood discoloring blue-stain fungus *Ophiostoma* sp. demonstrated copper tolerance up to 6 mM and some wood-rot fungi tolerated up to 10 mM of copper in solid medium. The reason of the high tolerance of our tested fungi may be the fact that they were isolated from copper treated wood and thus this habitat could have helped to elaborate their copper-tolerance.

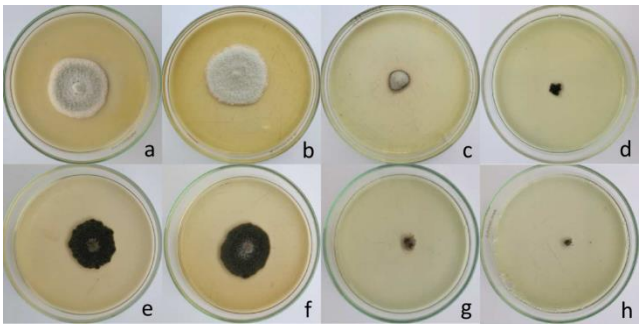

Fig. 3. Growth of *Alternaria tenuissima* (a, b, c, d) and *Ulocladium consortiale* (e, f, g, h) on PDA medium ammended with different copper sulphate concentrations, mM: a, e – 0 (control); b, f – 0.5; c, g – 3; d, h – 5

3.3. Cellulose biodegradation

Fungal enzymes enable them to grow on wood and use as a nutrient causing discoloration and mass lost resulting in biodegradation. The cellulose content of the sawdust used in our experiments made up 52.7 % of the whole mass. This is in agreement with the data of other authors indicating that cellulose represents about 45 – 50 % of wood weight [31]. We found that the copper based preservative did not suppress cellulose biodegradation by discoloration fungi *A. tenuissima* and *U. consortiale* and in some cases it seemed that the preservative could even encourage the degradation of sawdust (Fig. 4). Surprisingly, after 30 day cultivation *U. consortiale* reduced cellulose content more in the treated sawdust than in the untreated one, though the results did not change greatly after 90 days.

The greatest cellulose degradation was measured after 90 day cultivation of *A. tenuissima* on the untreated sawdust. The cellulose content decreased twice, compared with the initial content of cellulose. The decrease of cellulose content in the treated sawdust was less. Our results indicated that both discoloring strains studied were the degraders of the cellulose of the sawdust treated with the copper-based preservative, though the degree and the time of degradation differed. Certainly, the cellulose decay by

wood-rot fungi is considerably more efficient and can reach over 90 % [32]. Our results seem to be consistent with other researchers who found the loss of cellulose in wood by soft-rot fungi from 10 to 60 % [33].

3.4. Lignin biodegradation

The results in the Fig. 4 show the trend of lignin content decrease in all the experiment trials after cultivation of fungi studied. The initial content of lignin in the sawdust of scots pine was 39.03 %. This is in agreement with the data by Novaes et al. [34] who found that in different tree species the lignin content of wood can vary from 15 % to 40 %. The degradation of lignin in treated sawdust after 30 day incubation of fungi was weaker than in untreated. The significant reduce of lignin was noticed in both trials after 90 day cultivation of *A. tenuissima* and *U. consortiale*, as well. These results are consistent with data by Kluczek-Turpeinen et al. [35] on *Paecilomyces inflatus* strains that lignin loss was detected only after 12 weeks of incubation although decrease in spruce cellulose was detected already within 4 weeks.

Comparing the results of two strains studied, *U. consortiale* was a stronger lignin degrader than *A. tenuissima* though no significant difference was observed between the lignin content in the treated and untreated sawdust after 90 day cultivation.

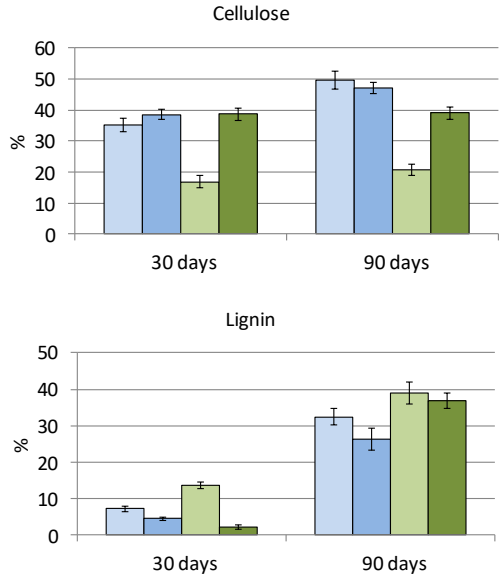

Fig. 4. Cellulose and lignin mass loss after 30 and 90 day cultivation of *Alternaria tenuissima* and *Ulocladium consortiale* in untreated sawdust (*A. tenuissima* – □, *U. consortiale* – □) and in treated with the copper based preservative sawdust (*A. tenuissima* – □, *U. consortiale* – □)

3.5. Production of cellulolytic enzymes

In microorganisms, the enzyme complement is complex and the concentration of various enzymes depends upon the species and is influenced by the culture medium [32]. Various factors and metals among them are known to affect the production of enzymes by filamentous fungi. In our experiment we analyzed the effect of copper based preservative on the activity of fungal lignocellulolytic enzymes. Cellulolytic enzymes play an important role in natural biodegradation processes in which plant lignocellulosic materials are efficiently degraded by fungi. Filamentous fungi produce a broad profile of cellulolytic enzymes [31, 36]. Among the cellulolytic enzymes endoglucanase is the major hydrolytic enzyme which initiates the attack in the amorphous regions of the cellulose fiber.

The fungal cellulase activity was measured during fungal growth on untreated and treated sawdust of pine with the copper based preservative. The results in Fig. 5 showed that the greatest endoglucanase activity was after *A. tenuissima* cultivation for 90 days on the treated sawdust. It's likely that copper based preservative stimulated endoglucanase activity of *A. tenuissima* but not of *U. consortiale*.

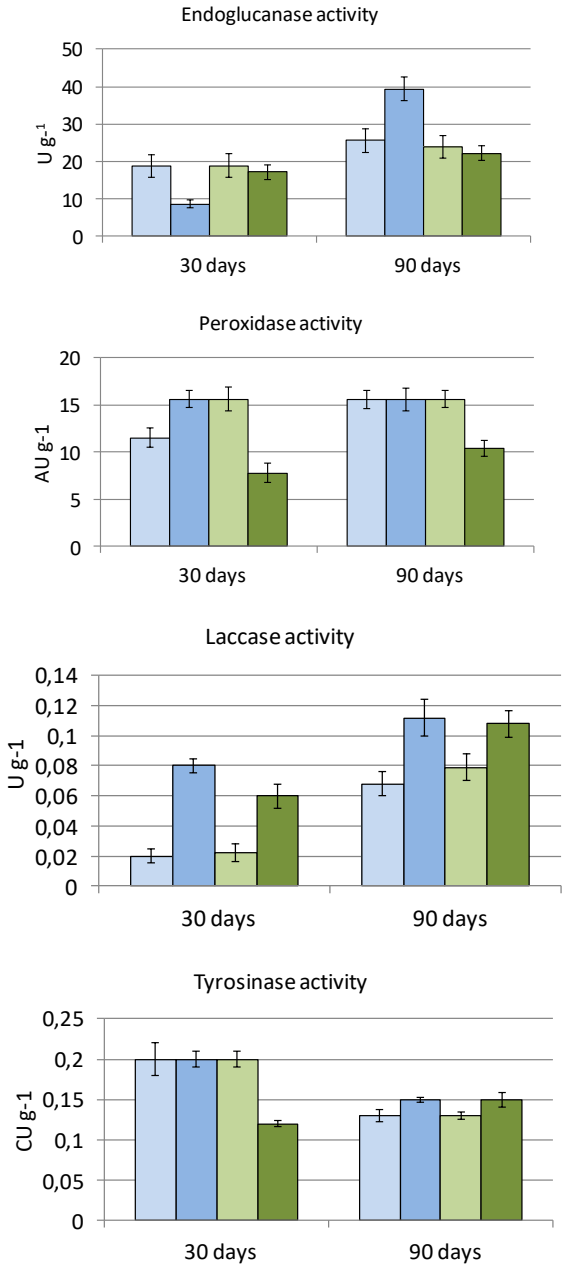

Fig. 5. Enzymatic activity of *Alternaria tenuissima* and *Ulocladium consortiale* on untreated sawdust (*A. tenuissima* – □, *U. consortiale* – □) and sawdust treated with the copper based wood preservative (*A. tenuissima* – □, *U. consortiale* – □) after 30 and 90 cultivation days

The present study demonstrated that the endoglucanase activity of *U.consortiale* after 30 day cultivation on the untreated and treated sawdust was similar (18.8 and 17.1 % respectively) and after 90 days of cultivation the endoglucanase activity increased slightly only in both cases in comparison with the 30 days. Ximenes et al. [37] ascertained that oligosaccharides are released during pretreatment, and hydrolysis of lignocellulosic biomass inhibits cellulose hydrolysis. In contrast, however, our results did not coincide with these findings.

Kluczek-Turpeinen et al. [35] highlighted the dependence of enzyme activity variation on time and substrates. He detected that during a prolonged incubation period of 12 weeks, endoglucanase activity of *Paecilomyces inflatus* declined gradually in compost, straw, and birch wood media, but remained rather stable on spruce wood media until the 12th week. Our results of *U.consortiale* endoglucanase activity were in agreement with these results.

There is little information about the physiology of the genus *Ulocladium*. Sallam et al. [38] reported about a high cellulase activity of *Ulocladium chartarum*. Pedersen et al. [36] ascertained that 50 different *Ulocladium* strains grown on wheat bran agar produced cellulose degrading enzymes, but their activity varied between species, as well as, between individual strains in each species. But *U. consortiale* was not mentioned among them.

Metals can be potent inhibitors of enzymes (or enzymatic reaction). Gadd [39] reported that copper decreased cellulase and amylase production by several fungi with a reduced enzyme activity correlating with an increasing metal concentration. Our results seemed to confirm these findings with one exception: *A. tenuissima* after 90 days of cultivation on treated sawdust.

3.6. Production of ligninolytic enzymes

Lignin degradation is a complex process and the ligninolytic enzymes have synergistic effects. In lignin degradation phenoloxidases, namely laccase and peroxidase, appeared to be the chief ligninolytic enzymes due to their higher secretion and stability [40].

Our results showed that in treated sawdust peroxidase activity remained at similar levels throughout 90 days of *A. tenuissima* cultivation (15.6 AU g^{-1}). However, over this period peroxidase activity of *U. consortiale* on the treated sawdust increased 1.3 times but was less than on untreated sawdust (Fig. 5). On the contrary, the change of peroxidase activity of *U. consortiale* on the untreated sawdust was not observed during the whole period of the experiment.

The differences of the enzymatic activity could be explained by the influence of heavy metals. Low concentrations of essential heavy metals are necessary for the development of the ligninolytic enzyme system. Baldrian [41] found that addition of low concentrations of Cu increased the activity of lignin peroxidase and Mn-peroxidase of *Phanerochaete chrysosporium*. The metals also increased solubilization and mineralization of lignin. Our results showed that heavy metals affected the peroxidase activity of fungi differently.

The research showed that the treated sawdust media appeared to be the preferred substrate for laccase production by both strains studied (Fig. 5). The most important result of investigation was higher laccase activity of both fungi studied in the treated sawdust after 30 and 90 of cultivation in comparison with the untreated sawdust.

Although in the treated sawdust laccase activity of *U. consortiale* was less than that of *A. tenuissima*, the actual lignin loss caused by *U. consortiale* was higher. The indirect correlation between laccase activity and lignin degradation was noted by Kluczek-Turpeinen [35] with *Paecilomyces inflatus* strain, as well. A possible explanation for this might be that copper is the cofactor of the enzyme laccase but lignin degradation depends on a whole complex of enzymes.

However, other authors [42] observed that the laccase activity was slightly inhibited by Cu^{2+}, though this enzyme is a copper-containing protein. This disagreement may be explained by the fact that dematiaceous fungi are less sensitive to copper effect because of the pigment melanin in the cell wall. Some fungal melanins are efficient bioabsorbers of copper and fungi produce them in response to copper [41].

Tyrosinase, common enzyme acting in melanin biosynthesis, is assumed to participate also in lignin degradation by the detoxification of its breakdown products [43]. This statement may explain the changes of tyrosinase activity during our experiment. *A. tenuissima* and *U. consortiale* showed different response in tyrosinase activity after 30 day cultivation but after 90 day cultivation the increase of tyrosinase activity was detected of both fungi studied on treated sawdust when lignin degradation was higher (Fig. 5).

The comparison of two dematiaceous fungi from wood discoloration showed that though morphologically these fungi were hardly distinguished, their enzymatic activity on the treated sawdust differed. *U. consortiale* carried out deeper cellulose degradation of the treated sawdust than untreated, meanwhile lignin degradation was higher in the untreated wood for both fungi. Moreover, *U. consortiale* was more activity in lignin degradation than *A. tenuissima*.

4. CONCLUSIONS

The present research helped to discover that the discoloration of treated wood was caused by two dematiaceous fungi *Alternaria tenuissima* and *Ulocladium consortiale.* The experimental data presented confirmed that these discoloring fungi were tolerant to the copper – able to grow up to 7 mM concentration of copper sulphate. Both fungi were capable of degrading lignin and cellulose while growing on sawdust treated with the copper-based anti-rot wood preservative. It's important to note that *U. consortiale*, as well as *A. tenuissima*, secreted higher levels of laccase and tyrosinase enzymes on the treated sawdust than on the untreated sawdust**.** The present study supplemented new information about *U. consortiale* which was little studied as a wood decomposer, function in lignocellulose degradation and metal tolerance.

The biochemical experiments on wood exposure to fungi provide information on the reasons why wood treated with preservatives could be vulnerable by dematiaceous fungi and the results could be used for selecting efficient copper concentration in creating new wood preservatives.

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