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Destructing Model Lignin Compounds by the Pioneering Strains of Fungi Colonizing Wood Wastes

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Abstract

Biological transformation routes were studied for some aromatic compounds by the strains of fungi *Trichoderma asperellum* and *Penicillium cyclopium* isolated earlier from hydrolytic lignin. It has been demonstrated that the destruction of these compounds is of oxidative nature. The biological transformation of aromatic substrates is accompanied by the reactions of α-oxidation, demethylation, oligomerization, aromatic ring destruction. Cellulase and Mn-dependent peroxidase were revealed to be present in the enzymatic complexes of the fungi. Correlation between the dynamics of Mn-dependent peroxidase activity and the rate of aromatic compound destruction has been observed. For composting lignocellulosic wastes, the strains *Trichoderma asperellum* Nos. 3, 10 and 11 those exhibit the maximal Mn-peroxidase activity and a high rate of aromatic substrate utilization have been chosen.

Key words: aromatic compounds, fungi, Mn-peroxidase activity

INTRODUCTION

The problem of environmental pollution with waste products of pulp-and-paper and resin industry belongs to a number of the main problems of the present. So, one of large-capacity waste products in Russia is presented by hydrolytic lignin (HL), whose structure includes lignin wood transformed to a considerable extent, polysaccharides difficult to hydrolyze, resinous substances, mineral and organic acids. One of the ways of HL recycling consists in obtaining organomineral fertilizers with the use of microorganisms and their enzymes. The microbiological method for recycling lignin-containing waste products represents the most environmentally appropriate process occurring slowly under natural conditions, for decades.

Being engaged in developing biotechnological processes for obtaining organomineral fertilizers (composts) on the base of HL, we have ascertained that for short-time recycling one should use microorganisms having enzymes of cellulase and oxidoreductase type. Only fungi are capable to a considerable extent for causing the biological destruction of lignocellulosic substrates, especially of their aromatic component difficult to transform. Earlier [1] we have demonstrated the potentiality for HL bioconversion by an association of microorganisms, capable for causing to degrade both low-molecular components (including those toxic for plants), and high-molecular components such as polysaccharides and lignin.

In the course of studying degradation succession occuring under composting HL, from the reference clamp we have separated 46 isolates of microorganisms most quickly have occupied the substrate [2]. These were the cultures of saprotrophic fungi those under natural conditions are capable of colonizing almost any organic substrate and to use its components as a nutrition source. Fast-growing imperfect

and ascigerous fungi belong to such microorganisms first colonizing wood substrata. Usually they feed on readily available substances of wood substrate, however, they are considered capable of finish the process of destruction up to completion [3], and therefore these fungi could appear promising fort composting.

According to the ability of grow on nutrient media with HL as a unique source of carbon, their growth rate and biomass accumulation, for the further studies we have chosen eight fungi cultures, such as *Penicillium cyclopium* Westling and *Trichoderma asperellum* Samuels, Lieckfelt *et* Nirenberg are selected. The fungi belonging to genera *Penicillium* and *Trichoderma,* are widely used as producers of cellulase and hemicellulase [4]. Some representatives of genus *Trichoderma* also exhibit biocide activity with respect to phytopathogenic fungi. According [5], with the incubation of *Trichoderma* sp. One can observe 100 % inhibition of *Botrytis* sp. and *Rhizoctonia solani*.

The studies on the fungi-induced degradation of lignin itself are difficult to carry out; therefore in the experiments we used compounds simulating the structural fragments of HL. Similar studies allow us to understand better the nature of decomposition processes inherent both in HL, and in native wood linins. The purpose of the present work consisted in the investigations concerning the biological transformation pathways for some aromatic compounds simulating the main lignin fragments and bonds with various types of ring substitution under the action of fungi strains *Trichoderma asperellum* and *Penicillium cyclopium,* in order to improve microbiological association in the processes of composting.

MATERIALS AND METHODS

The cultivation of fungi *T. asperellum* (strains Nos. 3, 4, 7, 8, 10, 11) and *P. cyclopium* Nos. 6, 9) carried out in Erlenmeyer flasks with a volume capacity of 300 mL at 26 °C. Into each flasks was poured 100 mL of liquid nutrient medium with the following composition, g/L: K_2HPO_4 1.5, $MgSO_4$ 0.21, NaCl 0.1, KNO_3 0.5, $FeSO_4$ 0.001, $CaCO_3$ 0.25, glucose 10.0. The inoculation was performed with the use of an agarised block of mycelium (0.5 cm in diameter). For the cultural filtrates, we determined the laccase activity through syringaldazine [6], the ligninase activity was determined by veratryl alcohol [7], and the Mn-peroxidase activity was determined through NADH [8]. The peroxidase activity was determined employing spectrophotometry (λ = 490 nm) from the oxidation of *o*-dianizidine at 20 °C; the cellulose activity was determined using filtering paper, hydrolyzing it in 0.1 M acetate buffer solution (pH 5.9) at 50 °C. The amount reducing sugars formed was determined using the Shomody–Nelson method [9]. The amount of protein was determined using the Lowry method [10] within range of 0.01–0.3 mg/mL. The measurements were carried out employing a SF-26 spectrophotometer (Russia). For unit activity we accepted the amount of enzyme necessary for the formation of 1 μmol of a product during 1 min per 1 mg of protein.

As model compounds, we used aromatic compounds with *o*-dihydroxylic, guayacylic, veratrylyc and syringilic type of substitution in the ring: vanillin, vanillic alcohol, protocatechoic acid, pyrocatechol (ReaKhim Co., Russia, chemical purity grade); guayacylpropanol-1, veratryl alcohol, lilac acid, synthesized *via* techniques presented in [11]. The purity of the reagents synthesized was verified using the method of high-performance liquid chromatography (HPLC).

The biological transformation was carried out in a liquid nutrient medium, adding a compound under investigation to the liquid nutrient medium in the course of inoculation with the concentration amounting to 0.04–0.08 mg/L. Samples were taken in the amount of 1 mL within the range of time from 1 to 24 days of cultivation, in 2 days each. The structure of metabolites was analysed *via* HPLC technique employing a Milichrom-1 chromatograph (Russia) which allows detecting the substances for the concentration values ranging within 0.1–0.001 %. The column was filled with Separon C_{18} sorbent, as the eluent we used 0.01 M KH_2PO_4 solution in 40 % methanol acidified with H_3PO_4 up to pH 3.9. The detection was carried out within ultra-violet spectral region at λ = 280 nm.

The identification of metabolites was carried out comparing their retention time values and spectral ratio values A_{260}/A_{230} with similar parameters for reference samples, as well as adding of a reference sample to a sample under investigation. The content of compounds in samples was calculated from the area of chromatographic peaks comparing with peak areas for samples with known concentration.

RESULTS AND DISCUSSION

The cellulase activity is observed for all the isolates being characterized by the presence of two maxima (ranging from 6th to 9th and from 14th to 16th days). One should mention especially *P. cyclopium* No. 6, whose cellulase activity is to a considerable extent (from 5 to 15 times) higher than the activity of other cultures (Fig. 1).

The determination of the oxidoreductase activity has revealed the presence of Mn-dependent peroxidase only. According the dynamics of Mn-peroxidase activity all the strains could be divided into two groups. Two distinct maxima of activity are inherent in the first group (*T. asperellum,* strains Nos. 4, 7, 10, 11; *P. cyclopium* Nos. 6, 9): ranging from 5th to 7th and from 13th to 15th days of cultivation (Fig. 2). The first maximum corresponds to the beginning the exponential growth phase, whereas the second maximum corresponds to the sporulation phase. For the second group of strains (*T. asperellum* Nos. 3, 8) three maxima of activity have been revealed at 8th, within the range from 13 to 14th and at 18th days. The maximum total Mn-peroxidase activity is exhibited by *T. asperellum* strains Nos. 8, 10, 11.

It is of common knowledge that Mn-peroxidase represents a key enzyme of some fungi and plays an important role in the decomposition of lignin [12]. The enzyme oxidizes phenolic units of lignin with the formation of phe-

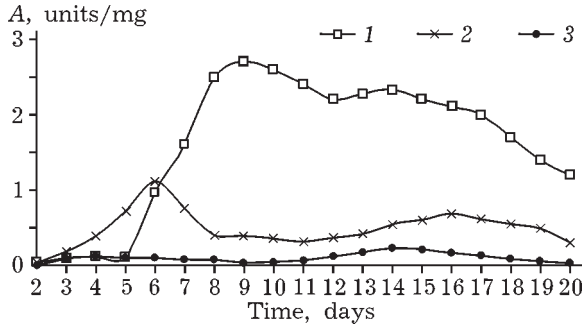


Fig. 1. Vatying the cellulase activity of *Trichoderma asperellum* No. 7 (*1*), *Penicillium cyclopium* No. 6 (*2*) and *Trichoderma asperellum* No. 11 (*3*) in the course of cultivation.

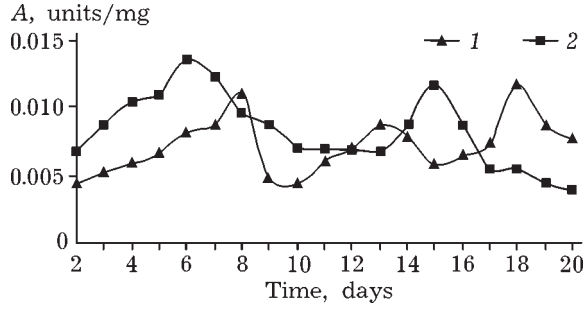


Fig. 2. Dynamics of Mn-peroxidase activity for strains *Trichoderma asperellum* Nos. 3 (*1*) and 11 (*2*).

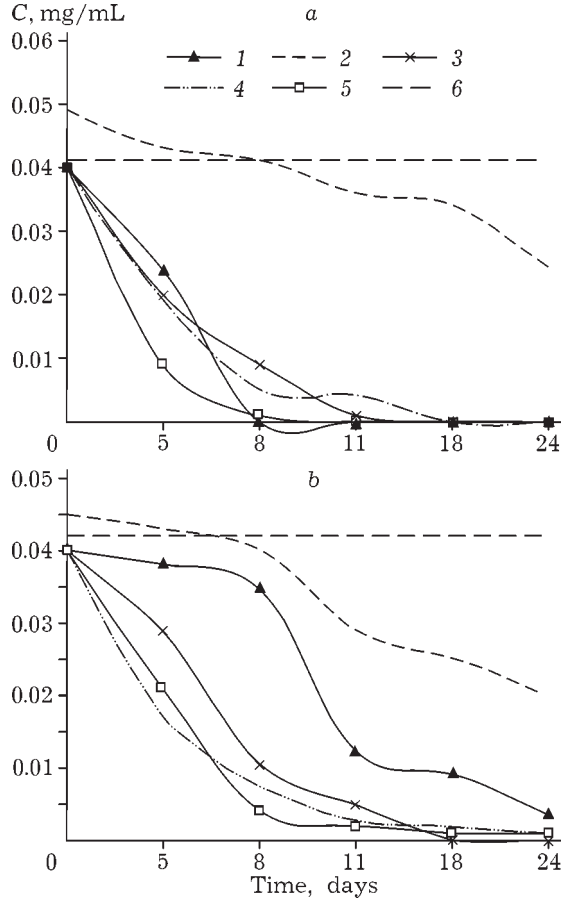


Fig. 3. Content of substrata under investigation in the cultural liquid of *Trichoderma asperellum* No. 8 (*a*) and *Penicillium cyclopium* No. 9 (*b*) in the course of cultivation: *1* – vanillin, 2 – veratrol, *3* – guayacylpropanol, *4* – vanillic alcohol, 5 – pyrocatechol, 6 – lilac acid.

noxyl radicals those, in turn, undergo various reactions resulting in depolymerisation [13]. By the example of model compounds, it is interesting to estimate the ability of the isolated cultures to destroy lignin.

All the cultures under investigation involved in the metabolism the compounds with free phenolic hydroxyl group. The maximum rate of substrata utilization was observed within the range from 3th to 5th days with complete disappearing them from 8th to 24th days depending on a strain. Figure 3 demonstrates the dynamics of substrata decay for the most and the least active strains. The lilac acid demonstrated the resistance with respect to the biological transformation by all the strains under investigation. Veratryl alcohol, the compound with substituted phenolic hydroxyl group, underwent the utilization with a much lower rate.

The decay of vanillin for *T. asperellum* strains Nos. 3, 8, 10, 11 was accompanied by appearing two dominating metabolites. In this case, for strains Nos. 3, 11 one of them has been identified as vanillic alcohol, whereas for strains Nos. 8, 10 it has been identified as protocatechoic acid. The second peak chromatographically coincided for all the four cultures and according to a combination data (spectral characteristics, double-wave detection $\lambda = 230/260$ nm) it could be hypothetically determined as a dimer of vanillic acid (Fig. 4). Hence, these isolates are capable of vanillin oxidation as well as of the subsequent dimerisation of the product formed. Despite the fact that the cultures belong to the same species of microorganisms,

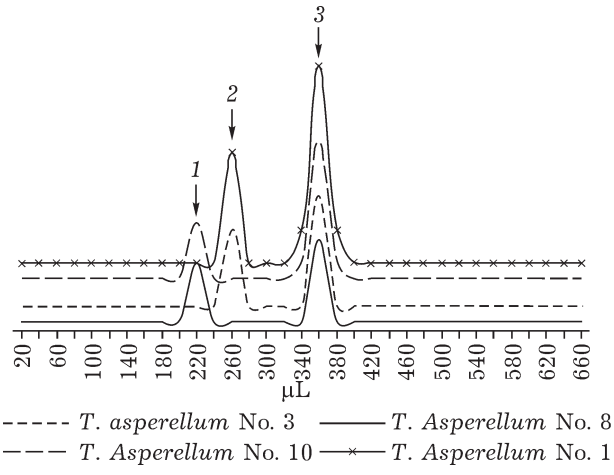


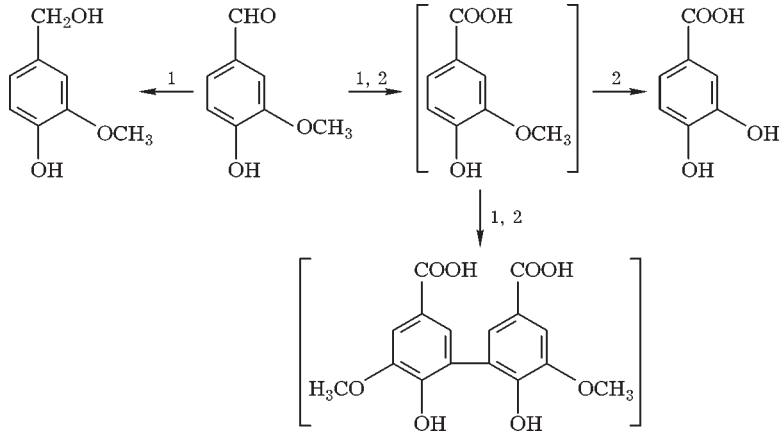
Fig. 4. HPLC profiles for vanillin metabolites (12 days of cultivationя): *1* – protocatechoic acid, *2* – vanillic alcohol, *3* – vanillic acid dimer.

there are strain differences manifested: some cultures (strains Nos. 3, 11) cause the reactions of reduction (1), whereas the others (strains Nos. 8, 10) cause demethoxylation reactions (2) (Scheme 1).

Both the ways are inherent in microbial decomposition [14].

The transformation of vanillin by fungi *P. cyclopium* (strains Nos. 6, 9), *T. asperellum* No. 7 was accompanied by the formation of a great number of compounds. Basing on the results obtained earlier [15] and the spectral analysis data, the assumption is made that the cultural liquid contains oligomerization products. For the majority of cultures, involving vanillic alcohol also occurred via oligomerization.

The ways of utilizing pyrocatechol by fungi have appeared also different. So, *P. cyclopium*



Scheme 1.

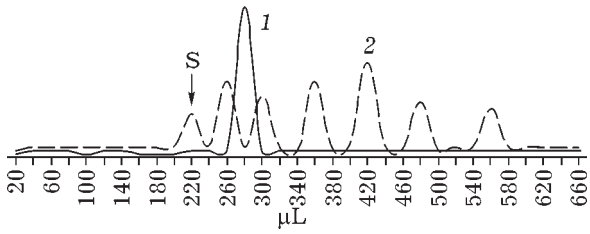


Fig. 5. HPLC profiles for the metabolites (S) of pyrocatechol incubated with fungal strains: *1* – *P. cyclopium* No. 9 and *T. asperellum* No. 7; *2* – *T. asperellum* (Nos. 3, 8, 10, 11).

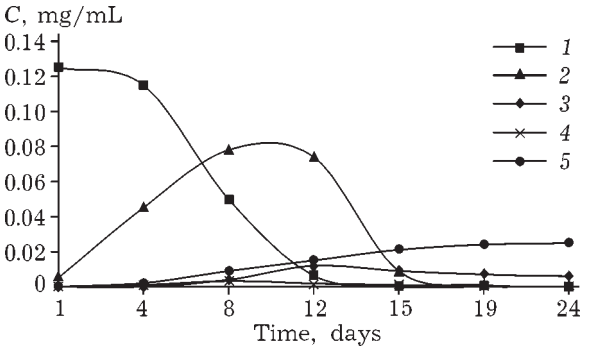


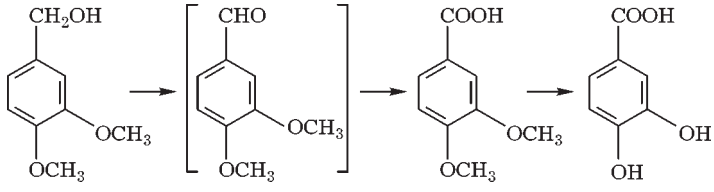
Fig. 6. Content of substrate (*1*) and metabolites (*2*–*5*) in the cultural liquid of *Trichoderma asperellum* No. 10: *1* – veratrol; *2* – veratric acid; *3* – vanillic alcohol; *4* – vanillin; *5* – protocatechoic acid.

No. 9 and T. *asperellum* No. 7 transformed this compound into a unique metabolite whose UV spectrum demonstrated an absorption maximum at 250 nm (Fig. 5). For the cultivation of strains *T. asperellum* Nos. 3, 8, 10, 11 we have revealed five to ten different products of aromatic nature. Strains *P. cyclopium* No. 6 and *T. asperellum* No. 4 by the 10th day have utilized pyrocatechol, to all appearance, to produce aliphatic compounds, since aromatic metabolites were not revealed.

Guayacylpropanol-1 was actively caused to undergo biological transformations (disappearance during 6–10 days) by three cultures only – *T. asperellum* Nos. 8 and *P. cyclopium* Nos. 6, 9; much more slowly (19–20 days) the utilization was stimulated by fungi *T. asperellum* (strains Nos. 4, 10, 11). In the first case the formation of a great of series aromatic metabolites was observed, whereas in the second case the utilization either was accompanied by the formation of single compound (for *T. asperellum* No. 11), or occurred with no formation of any aromatic substances.

Interesting data have been obtained for veratryl alcohol. It has been established that cultures *T. asperellum* Nos. 4, 7 and *P. cyclopium* No. 6 are not capable of utilizing the substrate under consideration. In the cultural liquid of *T. asperellum* No. 8 against the background of decreasing veratrol amount (27 % for 8 days) we have found out two new compounds such as veratric and protocatechoic acids. The biological transformation of veratrol into protocatechoic acid, to all apperance, proceeds stage-by-stage via the formation of veratric aldehyde, its subsequent oxidation up to veratric acid and further is accompanied by the demethylation of the latter (Scheme 2).

T. asperellum (strains Nos. 3, 10, 11) and *P. cyclopium* No. 9 more actively involved veratrol in metabolism. By the 20th day of cultivation *T. asperellum* No. 3 has utilized more than 90 % of a substrate to form four metabolites among those we have identified veratric acid disappearing by 12th day of cultivation. For *T. asperellum* No. 11 against the background of the active destruction of veratrol (60 % for 20 days) we have revealed only two metabolites with the prevalence of veratric acid and its amount gradually grew during all the experiment. *T. asperellum* No. 10 destructed veratrol faster than the others (Fig. 6). In this case, after different incubation time we revealed six metabolites in the cultural liquid among those we have identified veratric and protocatechoic acids, vanillic alcohol and vanillin (see Figs. 6, 7). Thus, the two parallel routes of veratrol destruction by strain *T. asperellum* No. 10 have



Scheme 2.

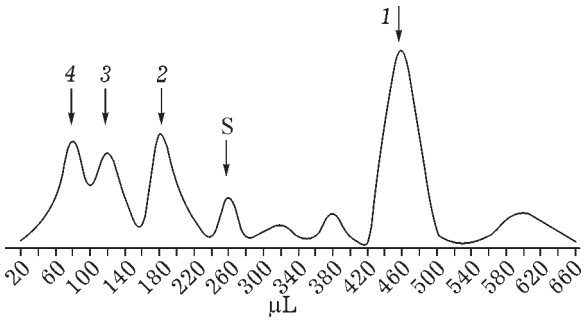


Fig. 7. HPLC profiles for the metabolites of veratrol (S), incubated with *Trichoderma asperellum* No. 10 for 12 days: *1* – veratric acid, *2* – vanillin, *3* – vanillic alcohol, *4* – protocatechoic acid.

been revealed: the first one occurs in a similar way as *T. asperellum* No. 8 (see Scheme 2); the second one consists in the transformation of veratrol into vanillic alcohol and partial oxidation of the latter to yield vanillin.

The analysis of Mn-peroxidase activity dynamics and of substrata decay has demonstrated that the active biological transformation of compounds with non-substituted phenolic hydroxyl group for all the cultures coincides with the first maximum of enzymatic activity. In the case of veratrol, for the cultures of the first group (two maxima of activity), the active destruction of a substrate coincides with the maxima of enzymatic activity, which was most distinctly observed in the case of fungi *P. cyclopium* No. 9 (Fig. 8). An exception is represented by fungi *T. asperellum* No. 11 tho se actively destroyed veratrol only after 13 days of cultivation. For the cultures of the second group (three maxima of Mn-peroxidase activity), starting the active veratrol decomposition corresponds to the second maximum for *T. asperellum* No. 3 and to the third maximum for *T. asperellum* No. 8.

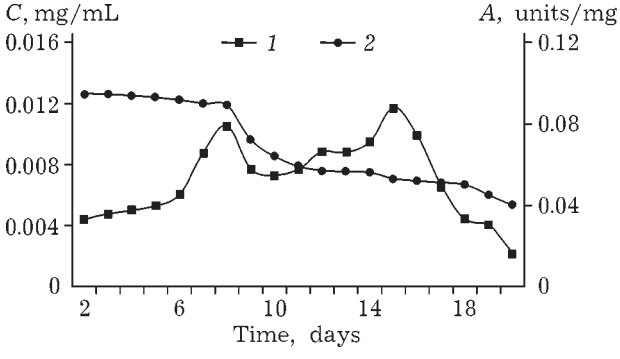


Fig. 8. Variation in the activity of Mn-dependent peroxidase (*1*) and in the content of veratrol (*2*) in the course of *Penicillium cyclopium* No. 9 cultivation.

CONCLUSION

Thus, all the strains under investigation actively involve in the metabolism the structures with free phenolic hydroxyl group. The main route of the biological degradation of these substances consists in oxidation, first of all, of α-atom in aliphatic chain. Some strains are capable for demethylation, oligomerization and aromatic ring destruction reactions. The etherification of hydroxyl groups causes involving the aromatic compounds in the metabolism of fungi to be complicated. All the strains of *T. asperellum* and *P. cyclopium* demonstrate not only cellulase-, but also Mn-peroxidase activity. The dynamics of Mn-peroxidase activity correlates with the rate of biological transformation for aromatic compounds. Among the cultures under investigation one could consider the most promising for composting lignocellulose waste products such fungi as *T. asperellum* (strains Nos. 3, 10 and 11), those exhibit a maximal Mn-peroxidase activity and a high rate of utilizing aromatic substrata.

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