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Transformation of Humic Substances of Highly Oxidized Brown Coal by Basidiomycetes *Trametes hirsuta* and *Trametes maxima*

O. I. Klein[a], N. A. Kulikova[a, b], A. I. Konstantinov[c], T. V. Fedorova[a], E. O. Landesman[a], and O. V. Koroleva[a]

[a] *A.N. Bach Institute of Biochemistry, Russian Academy of Sciences, Leninsky pr. 33, Moscow, 117071 Russia*
[b] *Department of Soil Science, Moscow State University, Moscow, 119991 Russia*
[c] *Department of Chemistry, Moscow State University, Moscow, 119991 Russia*
e-mail: klein_olga@list.ru

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Abstract—The ability of white rot basidiomycetes *Trametes hirsuta* and *Trametes maxima* to transform coal humic substances (HS's) under the conditions of solid phase cultivation in the presence or absence of an easily available source of carbon (glucose) has been studied. It was shown that during the growth of the fungal strains used in media containing HS's, destructive and condensation processes of HS transformation proceeded simultaneously. Based on a comparative physicochemical analysis of the initial HS's and HS's transformed by the fungi, it was established that, despite the introduction of glucose may favor a deeper transformation of HS's by basidiomycetes, the general direction of their modification is dominant reduction or oxidation and is determined by the physiological biochemical peculiarities of the strain used.

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INTRODUCTION

Since Coen and Gabriel for the first time showed in 1982 that fungi are able to grow directly on coal and are capable of metabolizing this substance [1], the processes of biological conversion of coal, first of all, brown coal, have become the subject of multiple investigations [2–7].

Recently, the interest towards the biological conversion of coal has been increasing steadily which is determined by the negative consequences for the environment emerging as a result of using coal as an energy source. This field is especially urgent for countries which are characterized by a high level of coal production, including Russia, which takes second place after the Federal Republic of Germany by this parameter. The production of brown coal comprises about 90 million tons per year in our country [8]. As compared to conversion by chemical or physical methods, microbiological and enzymatic approaches possess a number of advantages such as the lack of necessary setting of high temperatures and pressure [9].

Despite promising uses for the biological approach to the conversion of coal, its development requires an accurate selection of a microorganism, on the basis of which the creation of a bioconversion technique is being planned. This is determined by the peculiarities of coal as a substrate which includes a number of limitations for a suggested microorganism. One of the principal properties of coal is its toxicity, which complicates its biodegradation, is attributed to the presence of hard degradable compounds, for example, polycyclic aromatic hydrocarbons, as well as to the increased heterogeneity of the structure and solid phase state [8]. These indicated properties of coal, which are negative for its biotransformation, are responsible for the relatively small range of microorganisms which are capable of degrading coal; first of all, such microorganisms include basidiomycetes fungi, which produce lignolytic enzymes. These microorganisms belong to a small group of microorganisms which possess a unique system of extracellular ligninolytic enzymes and, due to this feature, are capable of degrading hard degradable substances with a complex phenol structure, such as lignin and coal. It was established that a leading role in this process belongs to the lignin degradation system, which includes enzymes of the ligninolytic complex: lignin peroxidase, Mn-peroxidase, and laccase, as well as low-molecular-weight secondary metabolites [10–14]. The efficiency of the lignin degradation process under the action of this system considerably depends on the availability of carbon sources. It was shown that the presence of an easily available carbon source in a nutrient medium intensifies the process of lignin biodegradation [14]. Nevertheless, the relationship between the efficiency of coal degradation and the composition of a medium remains unexplored.

The aim of the present study is to compare the biological degradation of brown coal under the action of producers of high active peroxidases and laccases—

Trametes hirsuta and *Trametes maxima* basidiomycetes—in complete nutrient media and in the absence of an easily available source of carbon (poor medium).

MATERIALS AND METHODS

T. hirsuta (Wuff.:Fr.) Pil. and *T. maxima* (Mont.) David & Rajchenb strains were used for experiments which were obtained from the collection of cultures of the Komarov Botanical Institute, Russian Academy of Sciences. In order to study the degradation of coal by the selected basidiomycetes strains, they were solid-phase cultivated using leonardite, i.e., coal of a high decomposition degree (Solntsevskii coal deposit, Sakhalin), according to [15].

During solid-state cultivation, 20 g of crushed leonardite with a particle size of ≤1 mm and 5 mL of complete or poor nutrient medium were placed in flasks of 750 mL in volume. During growing fungi in the complete medium, a nutrient medium of the following composition was used (g/L): glucose (10.0), peptone (3.0), $NaNO_3$ (3.0), KH_2PO_4 (0.6), $ZnSO_4 \cdot 2H_2O$ (0.001), K_2HPO_4 (0.4), $FeSO_4 \cdot 7H_2O$ (0.0005), $MnSO_4 \cdot 7H_2O$ (0.05), $MgSO_4 \cdot 7H_2O$ (0.5), $CaCL_2$ (0.5), $CuSO_4$ (0.25), pH 6.0. The poor medium without an easily available source of carbon had a similar composition, but it did not contain glucose. Afterwards, mycelium of fungi was planted (the inoculate volume is 20 mL). During the experiment, humidity was maintained at a level of 80–90% in the flasks. Coal was sterilized by autoclaving (120°C, 1 atm, 30 min). Cultivation was carried out for 30 days at 28°C.

The basic enzymes of the ligninolytic complex, i.e., lignin peroxidase, Mn peroxidase, and laccase, were monitored in order to estimate the functional state of the grown basidiomycetes.

Determination of lignin peroxidase activity. The activity of the enzyme was determined by the rate of veratric alcohol oxidation to veratric aldehyde by measuring the decrease in absorption at 310 nm in 0.1 M Na-tartrate buffer, pH 3.0 [16].

Determination of the Mn-peroxidase activity. The activity was assayed according to [17] using Mn^{2+} as a substrate. The oxidation of 1 μmoL of Mn(II) for 1 min was taken as a unit of activity.

Assay of laccase activity. The activity of laccase was determined spectrophotometrically at a wavelength of 410 nm using a 10-mM chromogenic catechol substrate in 0.1 M Na acetate buffer at pH 4.5 [18]. The increase in the optical density at one unit for 1 min was taken as a unit of activity. The rates of enzymatic reactions were recorded using PerkinElmer spectrophotometers (United States). In order to convert the enzymatic activities into international units (IU), standard units were normalized using the extinction coefficient of the substrate used.

In order to control changes in leonardite during cultivation of basidial fungi, after the termination of the experiment, humic substances (HS's) were extracted from it and their physicochemical properties were described. For this purpose, 0.1 M NaOH was added to leonardite at a weight ratio of 1 : 2, the pasty mass was quantitatively transferred into a dialysis bag with a pore size of 12–14 kDa and were dialyzed against distilled water for 48 h with multiple water replacements at a room temperature for the depletion of excess alkaline. HS's were then centrifuged for 20 min at 3000 g, and the supernatant was collected. The obtained solution of HS's was dried in a drying oven at a temperature of 50°C. HS's produced from leonardite, subjected to the same procedures (autoclaving, nutrient media addition, and incubation) but without the introduction of fungi, were used as a control sample. The principal physicochemical properties of the HS's were characterized according to parameters, such as the elemental composition, molecular mass (MM), and the group structure composition.

Elemental composition. An analysis of C, H, and N was carried out using an 1106 elemental analyzer (Carlo Erba Strumentazione, Italy). The content of oxygen was measured according to the difference between the mass of the sample and the total content of ash and CHN. The ash was determined by combustion of the preparation in a muffler at 850°C for 40 min.

Determination of the MM. The MM of HS's was assayed using exclusion chromatography according to the technique described in [19]. Fractionation of preparations was performed using a column filled with a Toyopearl-HW-50(S) gel (Japan); polysterol sulfonates with MMs of 4.48, 14.0, 20.7, 45.1, and 80.8 kDa were used as calibration substances (Polymer Standard Service, Germany). The concentration of HS's in the analyzed samples was found to be 40 mg/L. The mobile phase was made by phosphate buffer (0.028 M, pH 6.8); the elution rate was 1 mL/min. A UV detector was used in order to record HS's at the column outlet according to absorption at 254 nm. Average MMs were calculated using the GelTreat program [20].

Group structure composition. The group structure composition was analyzed by ^{13}C NMR spectroscopy. Specters were recorded using an Avance spectrometer (Bruker, Germany) at a retention time of 4 s. The indicated relaxation delay allows for the complete relaxation of carbon nuclei of almost all types which ensures the quantitativeness of ^{13}C NMR specters produced [21]. The concentration of HS's in the analyzed samples comprised about 30 g/L. The content of carbon in different structural fragments was determined by integrating the corresponding spectral regions

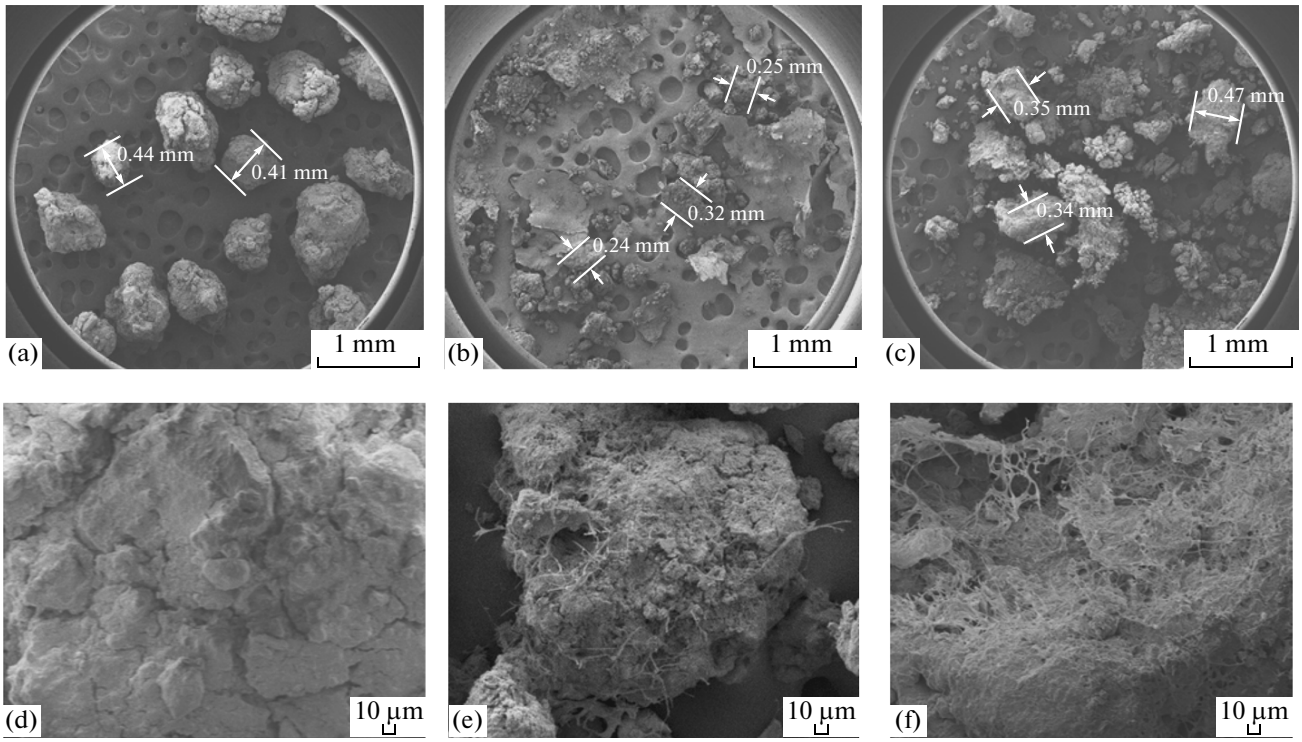

Fig. 1. SEM of leonardite samples after its interaction with *T. hirsuta* + *T. maxima* strains: (a, d) control, (b, e) medium without glucose, (c, f) medium with glucose, (a–c) ×25, and (d–f) ×300.

(ppm): 5–108 are atoms of C-unsubstituted of O- and N-substituted aliphatic fragments (ΣC_{Alk}), 108–165 are atoms of C-unsubstituted of O- and N-substituted aromatic fragments (ΣC_{Ar}), 165–187 are C atoms of carboxyl and ether groups (C_{COO}), 187–220 are C atoms of ketone and chinone groups ($C_{C=O}$).

Scanning electron microscopy (SEM). The SEM technique was used in order to visualize the changes in leonardite. After the termination of the combined cultivation of the studied strains, leonardite particles were dried to a constant mass at 50°C and mounted with a conductive double-sided adhesive sheet. In order to image the surface of coal, a high-resolution scanning electron microscope equipped with an autoemissive JSM-7500F cathode was used (JEOL, Japan). Images were obtained in a regime of low energetic secondary electrons, since this regime provided the highest resolution level. At the energy of a primary beam of 1 keV, the resolution was 1.5 nm; at 5 keV, 1 nm. Before microscopy, a metallic film was placed on the samples with a width of 5 nm using a magnetron spraying system.

RESULTS AND DISCUSSION

According to the SEM results, the analyzed strains of basidiomycetes actively interacted with leonardite that resulted in a marked loosening of its surface (Figs. 1a–1c), directly indicating the ability of fungi to degrade coal of a high decomposition degree using them as a source of nutrient substances. Previously, the ability of coal biosolubilization was demonstrated for basidiomycetes, such as *Gymnopus erythropus* [22], *Clitocybula dusenii* and *Nematoloma frowardii* [23], *Phanerochaete chrysosporium* [24], and *Trametes versicolor* and *Lentinula edodes* [25].

At a higher magnification (Figs 1d–1f), a system of hyphae is clearly seen which demonstrates the growth of the studied fungi with leonardite. In the medium without glucose, single hyphae are seen on the surface and hyphae grown inside leonardite particles, whereas in the complete medium, in which the growth of the fungus culture was more intense, mycelium formed a layer on the surface of coal. Intense surface growth of basidiomycetes did not allow for the visualization of their growth inside leonardite.

An analysis of the elemental composition of initial and transformed HS's of leonardite showed that the changes observed are only determined by the strain used but little depend on the presence of an easily available carbon source in the medium. In the case of *T. hirsuta* cultivation, changes in the carbon and hydrogen contents were hardly observed, while a decreased content of hydrogen and carbon in HS's was observed for *T. maxima* (Table 1). Taking into account that the nitrogen content hardly changed, one may suppose that *T. hirsuta* cultivation is accompanied by a partial reduction of HS's, cultivation of *T. maxima*, by their oxidation.

Table 1. Elemental composition and MMs of initial HS's and those transformed by the white rots *T. hirsuta* and *T. maxima*

Sample	Content, %				MM, kDa
	C	H	N	Ash	
Control	72.4	5.8	3.3	22.4	14.3
Medium without glucose					
T. hirsuta	75.3	5.9	3.6	22.5	26.9
T. maxima	70.4	5.1	3.1	20.2	28.5
T. hirsuta + *T. maxima*	69.5	5.1	2.9	19.9	25.1
Medium with glucose					
T. hirsuta	73.9	5.6	3.7	21.2	31.0
T. maxima	69.2	5.2	3.0	19.7	22.7
T. hirsuta + *T. maxima*	70.0	4.9	3.2	19.5	25.7

In order to validate the above-stated hypothesis, the O/C and H/C atomic ratios were calculated which allow for estimating the degree of oxidation and unsaturation of HS's. The results of the calculations presented in the Van Crevelen diagram (Fig. 2) show a bidirectional change in the HS properties. In the case of *T. hirsuta*, both studied atomic ratios decreased simultaneously; i.e., HS's were modified via the reduction and dehydration route in this basidiomycete strain. During the transformation of HS's under the action of *T. maxima* or in the case of co-cultivation of *T. hirsuta* and *T. maxima*, a decreased H/C ratio was also observed; at the same time, the O/C parameter was shown to increase; i.e., one may suppose that oxidative processes, such as carboxylation, proceed.

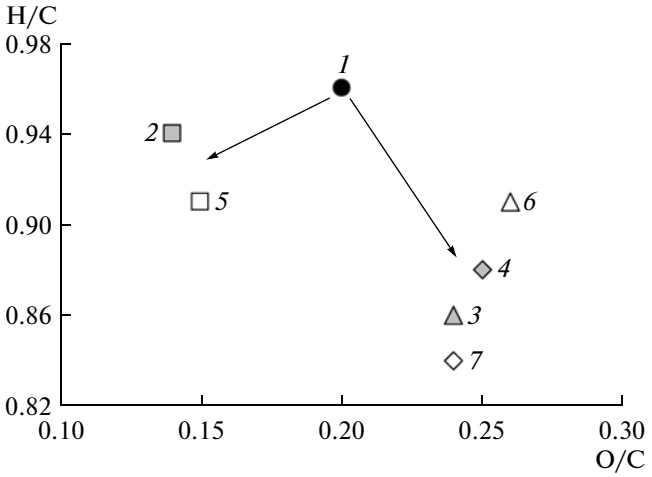

Fig. 2. Van Crevelen diagram for (*1*) initial leonardite HSs and those transformed by *T. hirsuta* and *T. maxima* fungi: *2–4*, a medium without glucose; *5–7*, a medium with glucose; *2* and *5*, *T. hirsuta*; *3* and *6, T. maxima*; *4* and *7*, *T. hirsuta* + *T. maxima*.

The relationship between HS transformation and the reduction capacity of fungi was previously demonstrated for the majority of basidiomycetes, including representatives of the genera *Polystictus, Hypholoma, Trametes*, and *Polyporus* [10]. Based on the screening results of more than 20 strains of fungi, only those strains were established to possess an ability to decolour HS solutions, in the presence of which the reduction of oxybenzoic and methoxybenzoic acids to primary alcohols is observed. Thus, reduction should be considered as a necessary stage in the degradation of HS's by basidiomycetes. It is supposed that cellobiose dehydrogenase may be responsible for the reduction processes, i.e., flavocytochrome capable of one electron reduction of aromatic radicals or two electron reductions of chinones with simultaneous oxidation of semiacetal sugar hydroxyl to produce carbonyl [26]. Therefore, the reduction of aromatic acids to alcohols may be regarded as a primary stage in the transformation of HS's by basidiomycetes.

A decreased H/C ratio was recorded for all studied samples which indicates a decreased percentage of aliphatic chains in the structure of HS's. It is of interest to note that the changes observed during HS modification in the presence of *T. hirsuta* are similar to the changes characteristic of conversion of fulvic to humic acids of soils, whereas the HS transformation under the action of *T. maxima* is similar to the processes which take place during the formation of fulvic acids from plant remains [27].

Increase in the MMs of HSs in all studied samples (Table 1) indicate that polymerization processes occur during HS transformation which are likely related to indirect enzymatic degradation based on the formation of radicals as main and by-products of enzymatic reactions with a further initiation of radical processes [28]. Another explanation for the recorded polymer-

Table 2. Group structure composition of initial HS's and HS's transformed by the white rots *T. hirsuta* and *T. maxima*

Sample	Content, %				$\Sigma C_{Ar}/\Sigma C_{Alk}$
	ΣC_{Alk}	ΣC_{Ar}	C_{COO}	$C_{C=O}$	
Control	31.7	46.0	18.3	4.5	1.5
	Medium without glucose				
T. hirsuta	27.7	52.8	15.3	4.2	1.9
T. maxima	28.3	45.3	18.1	8.3	1.6
T. hirsuta + *T. maxima*	19.9	56.1	18.6	5.4	2.8
	Medium with glucose				
T. hirsuta	26.5	47.6	18.3	7.5	1.8
T. maxima	21.2	53.8	18.9	6.1	2.5
T. hirsuta + *T. maxima*	28.1	42.8	16.7	12.5	1.5

ization of products of partial HS degradation may be such an ability of the ligninolytic enzymatic complex of selected strains as the presence of high-redox potential laccases, which as a rule possess condensing activity in relation to a wide range of phenolic substrates [28]. In any case, these data indicate the simultaneous proceeding of destructive and condensation processes in HS's during their growing with *T. hirsuta* and *T. maxima*. Similar differently directed processes were previously shown when studying the interaction between white rot fungi and lignin [26]. It was established that lignins of aspen and larch wood, transformed under the action of *Phanerochaete sanguine*, could have MMs that exceed the MMs of lingins in initial wood by 1.5 times. An analysis showed that condensation processes mainly go through C5 atom of the aromatic ring.

An analysis of the group structure composition of HS's by ^{13}C NMR spectroscopy (Fig. 3, Table 2) confirmed the previously established regularities and allowed for establishing more precise changes which occur in HS's during their interaction with the analyzed basidiomycete stains. It was revealed that the decreased content of carbon in HS's is mainly determined by a decrease in the relative content of aliphatic structure fragments for practically all samples. This is evidenced by both a decreased ΣC_{Alk} parameter and growth in the degree of $\Sigma C_{Ar}/\Sigma C_{Alk}$ aromaticity. *T. hirsuta* + *T. maxima* sample in a medium with glucose was an exception, for which the decreased percentage of carbon was attributed to a simultaneous decrease in the carbon content of both aliphatic and aromatic fragments that did not result in any increase in the $\Sigma C_{Ar}/\Sigma C_{Alk}$ aromaticity parameter. Therefore, one may suppose that when HS's are used as a source of nutrient substances by basidiomycetes, first of all, aliphatic chains are to be removed but not aromatic structures. A more marked decrease in the content of aliphatic groups in HS's during transformation by *T. hirsuta* and *T. maxima* strains was observed in the samples with a compete nutrient medium. This may be determined by both accelerated biomass growth and the resulting increased production of ligninolytic enzymes, and the use of glucose as a substrate for radical reactions.

During the cultivation of *T. hirsuta* in a medium without glucose, a decreased content of C_{COO} carboxyl group carbon in HS's was observed which agrees with the data on the elemental composition analysis showing a decrease in the oxygen content in HS's under these conditions. This confirms the hypothesis regarding the reduction of carboxyl groups to alcohol ones during the first stage of HS transformation. In the presence of glucose, the carboxyl group carbon content did not change, whereas that of ketone and chinone increased. This possibly indicates the presence of deeper HS degradation processes, which also include oxidation of alcohols, formed during the first stage of transformation, to ketones. The decreased HS oxidation level established by analyzing the elemental composition (Fig. 2) is likely determined by the loss of oxygen-substituted aliphatic fragments by HS's. Thus, the presence of easily available carbon source favors a deeper transformation of HSs by this fungal strain.

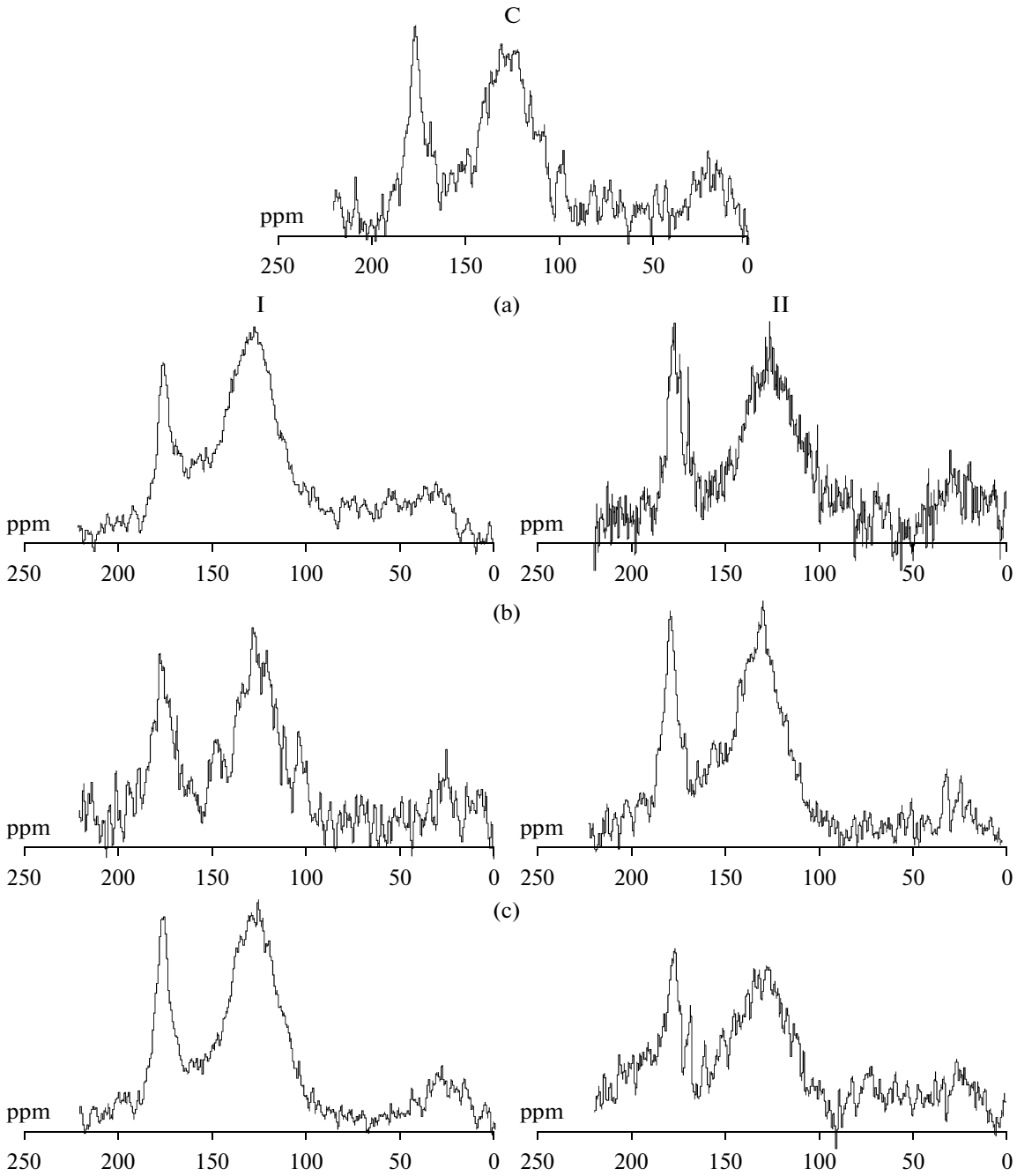

Fig. 3. ^{13}C NMR specters of initial HS's (C) and those transformed by (a) *T. hirsuta*, (b) *T. maxima,* and (c) *T. hirsuta* + *T. maxima*; I is a medium without glucose, and II is a medium with glucose.

During the transformation of HSs by *T. maxima* strain in a poor medium, the content of ketone and chinone group carbon was observed to increase at a simultaneous insignificantly decreased content of aliphatic, aromatic, and carboxyl fragments. Taking into account the total increase in the degree of oxidation according to the data of the elemental composition analysis (Fig. 2), one may suppose that an HS transformation occurred via a mechanism of alcohol group oxidation to produce ketones in this case. Since the content of carboxyl groups, which may serve as a source of alcohol groups, did not decrease in this case, aliphatic fragments became oxidized. In the presence of glucose, the $C_{C=O}$ content also increased; nevertheless, at the same time, the carboxyl group content also increased simultaneously. This indicates that, as in the case with *T. hirsuta*, the introduction of an easily available source of carbon leads to a more deep transformation of HS's.

Table 3. Comparative assessment of the lignin peroxidase, Mn-peroxidase, and laccase activities of the white rot fungi *T. hirsuta* and *T. maxima* during their cultivation with leonardite

Sample	Activity, IU		
	Lignin peroxidase, $\times 10^3$	Mn-peroxidase, $\times 10^3$	Laccase
	Medium without glucose		
T. hirsuta	5	18	0.2
T. maxima	6	8	0.3
T. hirsuta + *T. maxima*	3	7	0.1
	Medium with glucose		
T. hirsuta	15	10	0.2
T. maxima	4	8	0.2
T. hirsuta + *T. maxima*	10	14	1.9

Thus, the analysis of changes in the basic physicochemical properties of HS's during their transformation by the studied basidiomycete strains allows one to propose the following sequence of basic processes of HS degradation under the action of enzymes of the ligninolytic complex of basidiomycetes:

Aromatic acids $\xrightarrow{\text{reduction}}$ Alcohols $\xrightarrow{\text{oxidation}}$ Ketones Aldehydes $\xrightarrow{\text{oxidation}}$ Alyphatic acids

Monitoring of the enzyme activity in culture fluids showed that the dynamics in the activity of all three studied enzymes displayed a complex character and depended on the phase of culture development. Therefore, for comparison, the mean values of activities for the studied period of HS transformation were used (Table 3).

The data presented in Table 3 show that maximum laccase activity was observed in the ligninolytic complex of *T. hirsuta* and *T. maxima* which is typical of the strains used [15]. In the medium without glucose, the investigated strains possessed similar activities of lignin peroxidase and laccase but differed in the activity of Mn-peroxidase which was significantly higher than in *T. hirsuta*. It was previously shown that, under these conditions under the action of *T. hirsuta*, the content of carboxyl groups in HS's was shown to decrease (Table 2). Transformation under these conditions under the action of *T. maxima* did not lead to a decreased content of carboxyl groups but was favorable for growth in the content of ketone and chinone fragments in HS's. Therefore, one may conclude that, under the action of Mn-peroxidase, HS's became decarboxylated. In fact, correlation analysis showed a reverse linear dependence ($r^2 = -0.86$) between the activity of Mn-peroxidase which is found in the medium and the content of carboxyl groups in the HS's transformed (Fig. 4). The dependence obtained agrees with the data of other researchers which mentioned the decarboxylation of HS's under the action of fungal Mn-peroxidase which catalyzes the formation of a low-molecular-weight mediator with a high redox potential, i.e., chelated Mn(III) ion [23].

When grown in a complete nutrient medium, the activities of Mn- and lignin peroxidases were found to be higher in *T. hirsuta* than in *T. maxima*. The change in the enzyme profile resulted in that, during HS transformation, a more marked increase in the content of ketone and chinone groups was observed in *T. maxima*. Hence, one may suppose that the lignin peroxidase synthesized by *T. hirsuta* is responsible for the oxidation of alcohol groups in HS's to produce

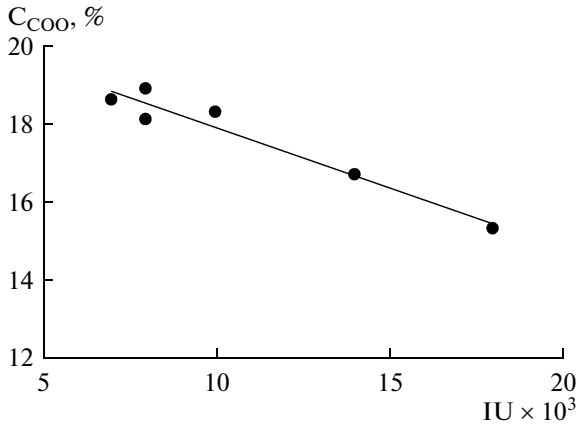

Fig. 4. Correlation field of variables: the Mn-peroxidase activity and the content of carboxyl group carbon C_{COO} of HS's transformed by *T. hirsuta* and *T. maxima*.

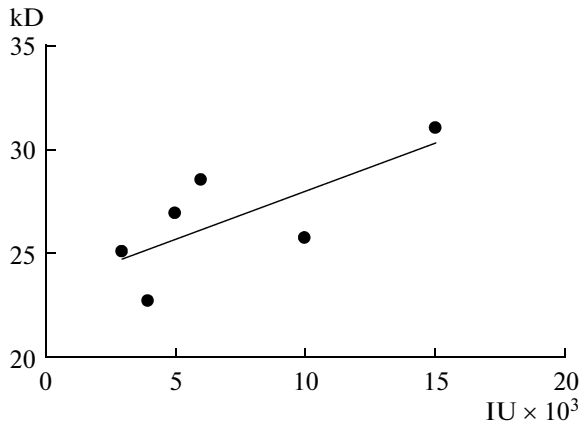

Fig. 5. Correlation field of variables: the MMs of HS's transformed by *T. hirsuta* and *T. maxima* and the activity of lignin peroxidase.

ketones that indicates the substrate specificity of lignin peroxidases [28].

It is interesting to note that a positive linear dependence ($r^2 = 0.73$) was observed between the activities of lignin peroxidase and the MM of HS's in the two media used (Fig. 5).

It is likely that the established effect of lignin peroxidase on the MMs of HS's indicates the involvement of this enzyme in the polymerization of products of partial HS degradation.

This study showed that in the case of *T. hirsuta* the presence of an easily available source of carbon mainly influences the ratio of lignin peroxidase and Mn-peroxidase in the produced multienzyme complex. In the absence of glucose, a higher activity of Mn-peroxidase was recorded; in the presence of this substance, that of lignin peroxidase. A change in the ratio of these two enzymes resulted in that the process of reduced decarboxylation of HS's, which dominates under the conditions of a poor medium and is likely the first stage in the transformation of HS's, changed into deeper processes of oxidative destruction leading to the formation of ketone fragments and aliphatic carboxylic acids. Despite the occurrence of oxidative processes during the transformation of HS's by the studied *T. hirsuta* strain in the medium with glucose and without, reduction processes dominated.

During growing *T. maxima*, the influence of an easily available source of carbon on the production of multienzyme complex components was less marked: the activity of Mn-peroxidase did not change, and the activity of lignin peroxidase decreased slightly upon the introduction of glucose into the nutrient medium. This resulted in fewer formed ketone groups in HS's and increased the content of carboxyl elements.

Thus, these results indicate that the presence of glucose in a medium favors a deeper transformation of HS's by the studied basidiomycete strains; however, the total direction of this modification: a predominant reduction or oxidation, is determined by the physiological-biochemical peculiarities of the strain. One may suppose that the domination of reduction processes during HS transformation was determined by the presence of extracellular cellobiose dehydrogenases in the composition of the complex. Nevertheless, in order to verify this hypothesis, additional research is needed.

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