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Comparative Analysis of the Ligninolytic Potential of Basidiomycetes Belonging to Different Taxonomic and Ecological Groups

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Abstract—Screening of the ligninolytic activity of basidiomycetes from the Komarov Botanical Institute Basidiomycetes Culture Collection (LE-BIN), Russian Academy of Sciences, belonging to different taxonomic and ecological groups was performed. The patterns of the position of taxa of active producers of ligninolytic enzymes in the modern system of fungi were identified. Cluster analysis showed that the group of fungi with the greatest ligninolytic and degradation potential includes representatives of the families Pleurotaceae, Polyporaceae, and Phanerochaetaceae, which perform the first stages of wood decomposition. As a result, species of interest for the further study of their oxidative potential and use in biotechnology were selected.

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Currently, the interest towards the bioconversion of lignocellulosic waste is steadily growing, which is determined by an increased volume of waste of the pulp and paper industry (PPI) and the woodworking industry and, as a consequence, an increased anthropogenic impact on the environment [1]. For example, according to the Federal State Statistics Service, the total amount of waste in the Russian pulp and paper industry has increased from 2991.2 million tons in 2005 to 4303.3 million tons in 2011. At the same time, the main methods for disposing of waste in our country remain warehousing in landfills and incineration after dewatering and compaction.

The use of biotechnological approaches for converting the pulp and paper industry's waste is limited by its toxicity due to the presence of compounds, such as polynuclear aromatic hydrocarbons, high concentrations of xenobiotics (dioxins, chlorine derivatives, etc.), and lignin, which is one of the most difficult biopolymers to degrade. That is why basidiomycetes—the most active lignin destructors—attract the attention of researchers as the key component of the lignocellulosic waste bioconversion technology. Of all known representatives of Basidiomycota, white-rot fungi are the most efficient lignin destructors [2]. The process of degradation of lignocellulosic substrates by these fungi consists of a large number of steps and depends on the presence of an enzymatic system (a unique ligninolytic complex consisting of laccase (EC 1.10.3.2) and various peroxidases, including the manganese (EC 1.11.1.13) and lignin (EC 1.11.1.14) peroxidases) in them [3–5]. It is known that some species of white-rot fungi (wood destructors) have a unique mechanism of detoxification of both lignin degradation products and various xenobiotics [6, 7]. For this reason, researchers constantly work to search for, isolate, and study new strains of basidiomycetes promising for use in bioconversion and bioremediation technologies [8–10].

This study is the first step in the development of a biotechnology for the processing of solid waste from the pulp and paper industry using basidiomycetes.

The goal of this study was to screen and assess the ligninolytic and degradation potential of white-rot fungi from different ecological and taxonomic groups and to select the most promising strains for use in the utilization of lignin-containing waste of the pulp and paper industry.

MATERIALS AND METHODS

Screening of cultures. More than 520 cultures of 330 species of higher (basidiomycete) fungi belonging to different taxonomic and ecological groups from the Komarov Botanical Institute Basidiomycetes Culture Collection, Russian Academy of Sciences (St. Petersburg), were used to assess the ligninolytic and degradation potential by rapid methods [11]. The most active strains shown in Table 1 were selected for further work. All cultures were stored in tubes on wort agar slants (wort 4%, agar 1.5%) at 4°C.

Species identification. The species of fungi were verified morphologically using conventional methods with subsequent genotyping.

Strains were grown on an MEA medium with 2% malt extract (Dia-M, Russia) and 1.6% agar (Difco,

Table 1. Characteristics of fungal strains studied

Strain	Species affiliation	Taxonomic affiliation	Geographic origin	Species ecology
LE-BIN 1998	*Antrodiella faginea* Vampola & Pouzar	Steccherinaceae, Polyporales	Russia, the Far East. The substrate is dry branches of deciduous trees	Secondary xylotroph (typical of heavily decayed wood or remaining on it for 15 years and longer), which causes white rot. Colonizes dead twigs, large branches, and trunks of deciduous trees, more rarely shrubs, of the genera *Alnus*, *Populus*, *Salix*, *Padus*, *Corylus*, and *Syringa*.
LE-BIN 0677	*Coriolopsis caperata* (Berk.) Murrill	Polyporaceae, Polyporales	Cuba	Xylotroph, which causes white rot. Lives on decomposed wood of angiosperms in serried forest communities and tropical mangroves.
LE-BIN 2047	*Lenzites betulina* (L.) Fr.	Polyporaceae, Polyporales	Finland, Lahti district. The substrate is a birch *Betula* sp. trunk in a coniferous forest	Wound primary xylotroph performing the first stage of wood decomposition (first seven years), which causes white rot. Lives on wood of deciduous trees, mostly on species of the genera *Betula*, as well as *Acer*, *Alnus*, *Carpinus*, *Corylus*, *Fagus*, *Fraxinus*, *Juglans*, *Prunus*, *Quercus*, *Salix*, *Sorbus*, *Tilia*, and *Ulmus*.
LE-BIN 2009	*Byssomerulius avellaneus* (Bres.) J. Erikss. & Hjortstam	Phanerochaetaceae, Polyporales	Russia, the Far East. The substrate is snag branches in an oak grove	Xylotroph, which causes white rot. Lives on dead and dead-and-down branches, as well as on litter of broad-leaved trees of the genera *Acer*, *Alnus*, *Carpinus*, and *Quercus*.
LE-BIN 0432	*Pleurotus ostreatus* (Jacq.) P. Kumm.	Pleurotaceae, Agaricales	Russia, Sochi	Primary wound xylotroph (performing the first stage of wood decomposition (first seven years), which causes mixed yellow rot. It lives on weakened and dying trees and dead branches and stumps of many deciduous and more rarely coniferous (Picea, Araucaria) tree species.
LE-BIN 2142	*Peniophora lycii* (Pers.) Höhn. & Litsch.	Peniophoraceae, Russulales	Russia, Rostov oblast. The substrate is cretaceous sediments in the steppe	Xylotroph, which lives on dead branches of various deciduous (*Acer*, *Fagus*, *Rhamnus*, and *Fraxinus*) and occasionally coniferous (*Pinus*) trees.
LE-BIN 1963	*Steccherinum murashkinskyi* (Burt) Maas Geest.	Steccherinaceae, Polyporales	Russia, the Far East, Kamchatka region. Found on a charred trunk of the birch *Betula platyphylla*	Secondary xylotroph typical of heavily decayed wood or remaining on it for 15 years and longer. Causes white rot. Lives on dead trunks and branches of various deciduous (*Acer*, *Betula*, *Populus*, *Salix*, and *Quercus*) and occasionally coniferous (*Abies*) trees.
LE-BIN 1911	*Trametes gibbosa* (Pers.) Fr.	Polyporaceae, Polyporales	Russia, Samara oblast, Stavropol district. Found on lime	Xylotroph, which causes actively spreading white rot. Lives on dead (mostly large-sized) wood (dead wood, stumps, snags, and treated wood) of deciduous (usually, on *Betula* and, more rarely, on *Alnus*, *Carpinus*, *Cerasus*, *Fagus*, *Populus*, *Salix*, *Tilia*, etc.) and, occasionally, coniferous (*Abies*, *Picea*) tree species. A highly competitive species able to slowly displace many species of wood-decaying fungi on the majority of substrates.
LE-BIN 1795	*Xerula radicata* (Relhan) Dörfelt	Marasmiaceae, Agaricales	Russia, Samara oblast, Stavropol district. At the base of a heavily damaged stump	Xylotroph, which develops on the roots (and possibly on the buried wood) of mostly deciduous trees (*Quercus*, *Fagus*). Fruiting bodies are usually found at the base of old heavily damaged tree stumps, which allows this species to be regarded as a secondary xylotroph.

United States) in Petri dishes (90 mm) at 25°C in the dark. The growth of the strains was characterized by the diameter of the colony on days 7 and 14 of growth. The characterization of the macromorphological and micromorphological features of the mycelium was performed for 4-week-old colonies by standard methods [12, 13]. The macromorphological characteristics included descriptions of the aerial mycelium, color, smell, and reversum of the colonies. The micromorphological description included the characteristic features of the hyphal system and the presence of swellings, buckles, and anamorphic elements (asexual

reproductive structures) on the mycelium. The teleomorph stage (sporocarp formation) in the culture was obtained on substrate blocks (birch sawdust with wheat bran, 3 : 1) in a Sanyo MLR-351H climatic chamber at 15°C and 85% humidity.

For genotyping the fungal strains, we performed partial (for 18S and 28S) and complete (for 5.8S) determinations of the rRNA nucleotide sequences, as well as the DNA sequences located between the genes encoding the 18S, 5.8S, and 28S rRNAs (ITS1 and ITS2 regions), using standard oligonucleotide primers to the conserved regions of the ribosomal genes: ITS1F 5'-CTT GGT CAT TTA GAG GAA GTA A-3' and ITS4B 5'-CAG GAG ACT TGT ACA CGG TCC AG -3 ' [14]. PCR was performed under the following conditions: 1 cycle of 5 min at 95°C and then 25 cycles (1 min at 90°C, 1 min at 56°C, and 1 min at 72°C), followed by 1 cycle of 10 min at 72°C.

Rapid method for assessing the total ligninolytic and laccase activities. For a qualitative assessment of the ligninolytic and laccase activity by the rapid method [11], the strains were grown in the dark at 25°C in Petri dishes (90 mm) in media of the following composition: 4.0% wort and 2.0% agar (S-A medium); (g/L): 0.6 KH_2PO_4, 0.4 K_2HPO_4, 0.5 $MgSO_4 \cdot 7H_2O$, 0.05 $CaCl_2$, 0.05 $MnSO_4$, 0.001 $ZnSO_4$, 0.0005 $FeSO_4$, 3.0 peptone, 10.0 g/L glucose, and 2.0 % agar (G-P medium); and G-P medium supplemented with 0.15 g/L $CuSO_4$ (G-P+Cu medium).

For inoculation, units of a 7-day-old mycelium (diameter, 5 mm) grown on an MEA medium were placed at the edge of a cup with the mycelial side facing down. The activity of the ligninolytic enzymes was determined after one and two weeks of growth. Mycelial blocks of 5 mm in diameter were cut near the edge of a growing colony and transferred into the wells of microbiological plates. Then, guaiacol (2 mL/100 mL H_2O) (Sigma, United States) and 1.0% syringaldazine (Sigma, United State) in C_2H_5OH were applied dropwise. The activity was evaluated visually by the color reaction intensity on a scale from "–" (no activity) to "+ + +" (the highest activity) at 5-, 15-, 30-, and 60-min and 3- and 24-h intervals.

Rapid method for the evaluation of lignin peroxidase activity. A qualitative assessment of the lignin peroxidase activity was performed by a rapid diffusion method [15]. Strains were grown in the dark at 25°C in Petri dishes (90 mm) on S-A, G-P, and G-P+Cu media supplemented with 0.02% Azure B under sterile conditions before aliquoting. For inoculation, blocks of 7-day mycelium (diameter, 5 mm) were placed on an MEA medium at the edge of a dish with the mycelial side facing down. The lignin peroxidase activity was determined after one and two weeks of growth. The activity was assessed visually by the intensity of changes in the dye color on a scale from "–" (no activity) to "+ + +" (the highest activity).

Cultivation methods. Inocula were grown in 750-mL stationary Erlenmeyer flasks with porcelain beads on an agar-free G-P medium at 25°C for 10–14 days depending on the growth rate of the fungus. Before inoculation, the inoculum was crushed with porcelain beads at 180 rpm for 20 min to form a homogeneous suspension, which was added to the cultivation flasks to a final volume of 10% under sterile conditions.

Submerged cultivation was performed on a shaker at 180 rpm for 20 days in 750-mL conical flasks containing S-A, G-P, and G-P+Cu media without agar. Sampling of the culture liquid for measuring the laccase activity was performed daily.

Determination of laccase activity. The laccase activity was determined spectrophotometrically by the increase in absorbance at $\lambda = 525$ nm after adding 0.018 mL of 0.1% alcoholic solution of syringaldazine to the reaction mixture (1.7 mL of phosphate–citrate buffer (pH 5.0) and 0.2 mL of culture filtrate) [16]. The increase in absorbance in 1 mL of the reaction mixture for 1 min was taken as one unit of enzymatic activity.

Isolation of DNA. Fungal chromosomal DNA was isolated from the mycelium on days 8–10 of surface culturing. The mycelium was washed with distilled water and then dried between layers of filter paper. The dried mycelium was ground in a mortar with liquid nitrogen to obtain a homogeneous white–yellow powder. DNA was isolated from the ground fungal biomass using DNeasy Plant Mini Kit (50) protocols (Qiagen, United States) according to the protocol provided by the manufacturer. The purity of the samples was monitored by agarose gel electrophoresis. The resulting chromosomal DNA samples were stored at –20°C.

Isolation of total RNA. Total RNA was isolated from the ground fungal biomass using a RNeasy Plant Mini Kit (50) (Qiagen, United States) according to the protocol provided by the manufacturer, adapted for plant material and filamentous fungi. Total RNA was used as a template for the synthesis of the first cDNA strand in the reverse transcription reaction.

Reverse transcription. A reverse transcription reaction (RT) was performed using a Mint reagent kit for cDNA synthesis (Evrogen, Russia) according to the manufacturer's instructions. The first cDNA strand was amplified in a PCR reaction to obtain double-stranded cDNA (ds-cDNA) enriched in full-length sequences. The resultant ds-cDNA was used as a template for PCR.

PCR. The chromosomal DNA and ds-cDNA of each test fungus was used as a template for PCR. PCR was performed in a reaction mixture of the following composition: 2 μL of 10 X Encyclo buffer (Evrogen, Russia), 0.4 uμL of 50 X mixture of dNTP (20 mM each) (Evrogen, Russia), 1.0 μL of forward and reverse primers (0.5 μM), 0.4 μL of 50 X Encyclo polymerase (Evrogen, Russia), 1 μL of template DNA, and 14.2 μL of sterile water. The reaction was performed in a C1000 thermocycler (Bio-Rad, United States). The amplification products were separated by electrophoresis in a 1.2% agarose gel. The results were visualized under a UV transilluminator at 260 nm.

Laccase gene identification. The presence of the laccase gene was detected by PCR using the following program: 1 cycle of 5 min at 94°C, then 35 cycles (15 s

at 90°C, 20 s at 56°C, and 30 s at 72°C), followed by 1 cycle of 10 min at 72°C. The PCR fragments were amplified using the following degenerate primers:

LacA1 For 5'-CAYTGGCAYGGWTTYTTYCAR-3'

LacA1 Rev 5'-RTGRAARTCDATRTGRCARTG-3'

LacA2 For 5'-GGNACNTTCTGGTAYCAYAGYCAY-3'

LacA2 Rev5'-RTANCGNAGDATAGCVGAGTT-3'.

Purification and cloning of PCR fragments. PCR products were purified using a QIAquick Gel Extraction Kit (50) (Qiagen, United States) according to the protocol suggested by the manufacturer. The isolated PCR fragments were cloned using the pAL-TA vector for the rapid cloning of PCR products (Evrogen, Russia) in *Escherichia coli* XL1-Blue competent cells.

Determination of nucleotide sequences. The nucleotide sequences of the cloned DNA fragments were determined using an ABI PRISM® BigDye™ Terminator v. 3.1 reagent kit with the subsequent analysis of the reaction products in an ABI PRISM 3730 automatic DNA sequencer (Applied Biosystems, United States) at the Genome Collective Use Center (Engelhardt Institute of Molecular Biology, Russian Academy of Sciences).

Sequence analysis. A homology search in GenBank was performed using the BLAST server (http://www.ncbi.nlm.nih.gov/BLAST) [17]. The amino acid and nucleotide sequences were edited using the BioEdit Sequence Alignment Editor (http://www.mbio.ncsu.edu/bioedit/bioedit.htmL). Alignment of the amino acid sequences of proteins was performed using the ClustalW program (http://www.ebi.ac.uk/clustalW).

Statistical data processing. Three biological and analytical replicates were performed for all experiments. A statistical analysis of the results was performed using the STATISTICA 8.0 program. To combine the fungal strains into groups, the experimental data were subjected to tree clustering. For this purpose, the preliminary screening results were scored. To form clusters, the Euclidean distance was used as the distance between objects. The clusters were combined using the neighbor-joining method (single bond).

RESULTS AND DISCUSSION

Screening and identification of the species affiliation of cultures. We studied the cultural and morphological characteristics of the studied strains to determine their taxonomic affiliation (Table 1). It was found that the cultural traits of strains *Antrodiella faginea* 1998, *Coriolopsis caperata* 0677, *Lenzites betulina* 2047, *Pleurotus ostreatus* 0432, *Peniophora lycii* 2142, *Trametes gibbosa* 1911, and *Xerula radicata* 1795 were typical of these species [12, 18]. The morphology of the vegetative mycelium of strains *Byssomerulius avellaneus* 2009 and *Steccherinum murashkinskyi* 1963 (Figs. 1a, 1b) was studied for the first time and allowed us to perform their identification, which was confirmed using the basidiomes obtained from the culture (Fig. 3a, 3b). To confirm the correctness of strain identification, we performed partial (for 18S and 28S) and complete (for 5.8S) sequencing of their rRNA, as well as the ITS1 and ITS2 regions (Table 2). The species affiliation of strains 1998, 0677, 2047, 0432, 2142, 1911, and 1795, determined by the morphological traits, coincided with the results of DNA typing (100% homology). In the case of strains 1963 (*Steccherinum murashkinskyi*) and 2009 (*Byssomerulius avellaneus*), the resulting sequences did not coincide completely with any of the sequences in the database. The nucleotide sequence of basidiomycete *Byssomerulius avellaneus* 2009 had 98% homology with that of *Phlebia chrysocreas* (gi no. 37622321); distinctions were observed in the ITS1 and ITS2 variable regions and in the 28S ribosome region. In the case of *Steccherinum murashkinskyi* 1963, the highest homology (95 and 94%, respectively) was found for *Steccherinum ochraceum* (accession 157057321) and *Steccherinum litschaueri* (gi no. 56181683), which allowed us to classify the studied strain with *Steccherinaceae* sp. (Taxonomy ID: 81060). The sequences of 18S rRNA, 5.8S rRNA, and 28S rRNA of the fungi *Phanerochaete avellanea* (synonym *Byssomerulius avellaneus*) and *Steccherinum murashkinskyi* were added in the NCBI under the accession numbers GU062467 and FJ798705, respectively.

Thus, the group of strains studied included representatives of aphyllophoroid xylotrophic fungi of the families Phanerochaetaceae (*P. avellanea*), *Steccherinaceae* (*A. faginea* and *S. murashkinskyi*), and Polyporaceae (*C. caperata, L. betulina* and *T. gibbosa*), belonging to the order Polyporales; agaricoid fungi of the families Marasmiaceae (*Xerula radicata*) and Pleurotaceae (*P. ostreatus*), belonging to the order Agaricales; and the family Peniophoraceae (*P. lycii*), belonging to the order Russulales.

To select the most promising strains, we used a traditional technique of screening for ligninolytic activity. The total ligninolytic, laccase, and lignin peroxidase activities were determined using guaiacol [19] syringaldazine [20] and azure B [15], respectively, as model substrates. The bleaching of polyphenol pigments, including azure B, by basidiomycetes may also indicate the presence of peroxidase and H_2O_2-producing oxidases [8]. As was shown earlier, the ability of some members of aphyllophoroid xylotrophic fungi to bleach polymeric dyes correlates with their ability to degrade various xenobiotics, which allows the Azure B bleaching test to be used in search for fungi with a high detoxification potential [8].

The results of a qualitative assessment showed that the studied strains exhibited a high ligninolytic and laccase activity after one or two weeks of growth in all media (Table 3). The composition of the culture medium had a significant effect both on the growth of the fungi studied and on the production of extracellular ligninolytic enzymes by them. Natural wort (S-A medium), used in the experiments, is a rich organic medium and is best suited for the cultivation of basidiomycetes. In addition, it also contains many natural organic compounds that can function as inducers of

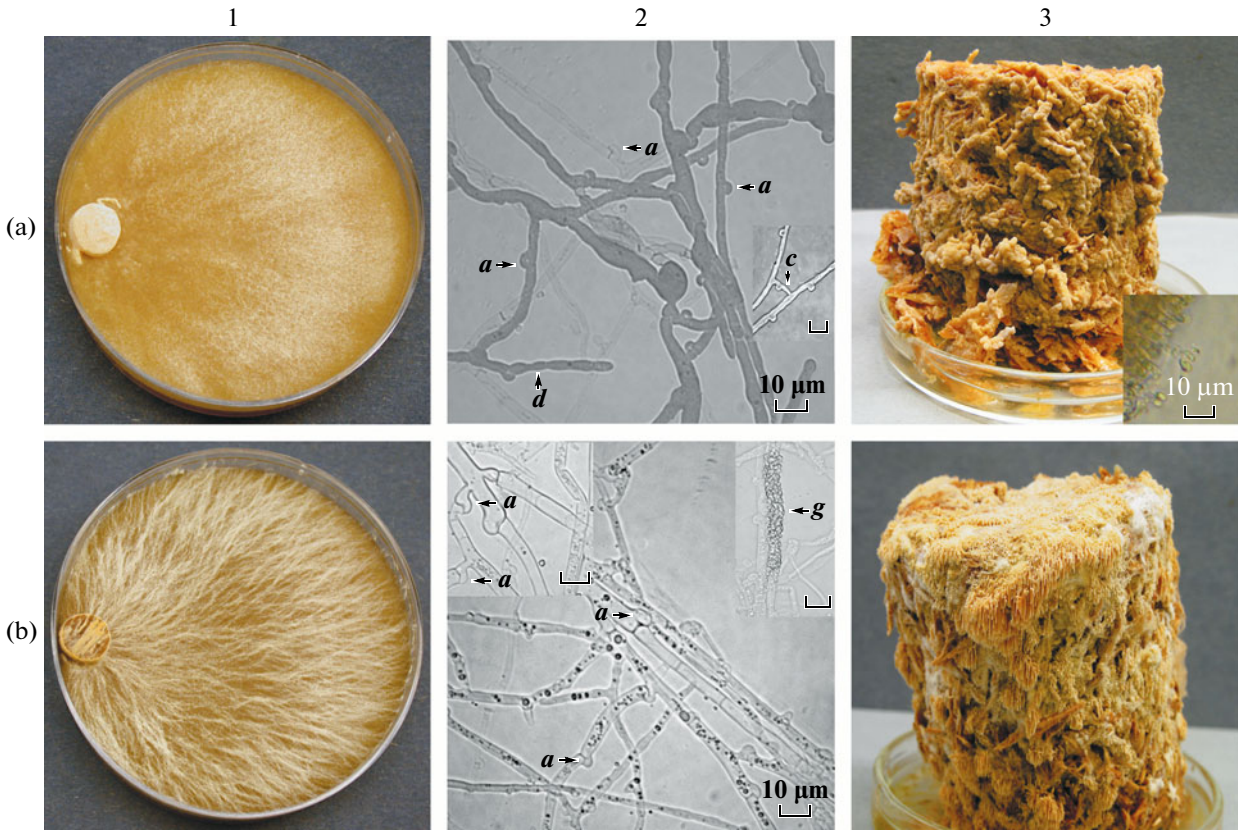

Fig. 1. White-rot fungi (a) *Byssomerulius avellaneus* 2009 and (b) *Steccherinum murashkinskyi* 1963: (1) surface growth of the fungi on an S-A medium; (2) micromorphology of fungal mycelium, 1000; (3) fruiting on sawdust blocks. Arrows and Latin letters designate (*a*) clamp connections, (*c*) anastomoses, (*d*) gloeocystidia, and (*g*) hypha incrustation.

ligninolytic enzymes. Semisynthetic medium G-P, containing glucose as a carbon source and peptone as a nitrogen source, on the one hand, does not contain natural inductors. On the other hand, when grown on glucose-containing media, white-rot fungi are able to synthesize glucose oxidase (EC 1.1. 3.4), which is a major source of H_2O_2 in some basidiomycetes during growth on wood substrates [21]. To stimulate the biosynthesis of the ligninolytic enzyme laccase, the G-P medium was supplemented with $CuSO_4$ (medium G-P+Cu). The S-A medium was more advantageous for strains *A. faginea* 1998, *L. betulina* 2047, *P. avellanea* 2009, *P. ostreatus* 0432, and *X. radicata* 1795. On the G-P medium, the fungi *C. caperata*, *P. avellanea*, *P. ostreatus*, *S. murashkinskyi*, and *T. gibbosa* grew more slowly: the Petri dish was completely covered

Table 2. Verification of the species affiliation of fungal strains by DNA typing

Strain	The closest strain from the NCBI database (Taxonomy ID)	Identity, %
Antrodiella faginea 1998	*Antrodiella faginea* (92699)	99
Coriolopsis caperata 0677	*Coriolopsis caperata* (195176)	100
Lenzites betulina 2047	*Lenzites betulinus* (5632)	99
Byssomerulius avellaneus 2009	*Phanerochaete avellanea,* synonym *Byssomerulius avellaneus*, (194676) GU062467	100
Pleurotus ostreatus 0432	*Pleurotus* sp. *'Florida'* (188765)	99
Peniophora lycii 2142	*Peniophora lycii* (154539)	100
Steccherinum murashkinskyi 1963	*Steccherinum murashkinskyi* (627145)FJ798705	100
Trametes gibbosa 1911	*Trametes gibbosa* (160864)	100
Xerula radicata 1795	*Hymenopellis radicata,* synonym *Xerula radicata* (937743)	100

Table 3. Activity of the ligninolytic enzymes of studied and control fungal strains determined by a rapid method

Strain	Medium	Duration of cultivation, days	Growth, mm	Activity		
				guaiacol	syringaldazine	azure B
LE-BIN 1998	S-A	7	33.3 ± 1.1	+	++	–
		14	78.7 ± 0.9	++	++	+
	G-P	7	36.3 ± 1.1	±	+	–
		14	89.3 ± 0.9	±	±	±
	G-P+Cu	7	23.3 ± 4.4	±	++	–
		14	77.3 ± 1.8	+	+	–
LE-BIN 0677	S-A	7	71.0 ± 2.7	+++	+++	–
		14	>90.0	++	++	+
	G-P	7	37.0 ± 2.0	++	++	–
		14	75.3 ± 1.8	+	+	–
	G-P+Cu	7	46.3 ± 2.9	+++	+++	–
		14	70.6 ± 1.8	++	++	–
LE-BIN 2047	S-A	7	78.7 ± 0.9	++	++	+
		14	>90.0	+++	+++	+++
	G-P	7	73.7 ± 2.2	±	±	–
		14	>90.0	±	±	±
	G-P+Cu	7	56.7 ± 3.1	+	++	–
		14	>90.0	+	++	–
LE-BIN 2009	S-A	7	56.3 ± 2.4	+	++	–
		14	88.3 ± 2.2	++	++	±
	G-P	7	26.7 ± 2.4	++	++	–
		14	69.3 ± 3.8	±	±	–
	G-P+Cu	7	37.3 ± 0.9	±	±	–
		14	78.7 ± 0.9	+	–	–
LE-BIN 0432	S-A	7	75.0 ± 1.3	++	+++	±
		14	>90.0	++	++	+++
	G-P	7	42.3 ± 1.1	±	±	–
		14	63.0 ± 2.0	±	±	–
	G-P+Cu	7	60.7 ± 1.8	+	+	–
		14	>90.0	±	+	±
LE-BIN 2142	S-A	7	25.0 ± 0.1	++	+	–
		14	64.3 ± 3.8	+	+	±
	G-P	7	51.0 ± 0.3	±	±	–
		14	87.3 ± 0.4	±	±	–
	G-P+Cu	7	40.3 ± 1.8	+	++	–
		14	80.0 ± 0.1	++	+	–
LE-BIN 1963	S-A	7	34.7 ± 1.8	++	++	–
		14	68.3 ± 4.4	+++	+++	±
	G-P	7	23.7 ± 1.1	++	+++	–
		14	47.0 ± 1.3	++	++	–
	G-P+Cu	7	17.7 ± 0.4	+++	+++	–
		14	34.7 ± 0.4	+++	+++	–
LE-BIN 1911	S-A	7	73.0 ± 1.3	++	++	–
		14	>90.0	+++	++	+++
	G-P	7	34.7 ± 0.4	++	+++	±
		14	72.0 ± 2.0	±	+	+
	G-P+Cu	7	35.0 ± 2.0	+++	+++	–
		14	81.7 ± 2.4	++	+++	±

Table 3. Contd.

Strain	Medium	Duration of cultivation, days	Growth, mm	Activity		
				guaiacol	syringaldazine	azure B
LE-BIN 1795	S-A	7	43.0 ± 0.5	±	–	–
		14	>90.0	+++	+++	–
	G-P	7	45.0 ± 1.34	+	+	–
		14	87.3 ± 1.8	++	+	±
	G-P+Cu	7	40.3 ± 1.8	+	++	–
		14	81.7 ± 2.4	+++	++	–
Trametes hirsuta 072*	S-A	7	>90.0	++	+++	–
		14		++	+++	++
	G-P	7	65.7 ± 0.9	+++	+++	–
		14	>90.0	++	++	±
	G-P+Cu	7	78.3 ± 1.0	+++	+++	–
		14	>90.0	+++	++	–
T. maxima 0275*	S-A	7	78.0 ± 1.3	+++	+++	+
		14	>90.0	+	+	+++
	G-P	7	55.3 ± 3.1	+	++	–
		14	84.3 ± 0.9	±	±	±
	G-P+Cu	7	65.5 ± 2.56	++	++	–
		14	85.5 ± 1.5	++	++	–
T. ochracea 093*	S-A	7	67.0 ± 2.7	++	++	–
		14	>90.0	++	++	+++
	G-P	7	50.7 ± 0.9	±	±	–
		14	84.3 ± 1.6	±	±	–
	G-P+Cu	7	54.7 ± 2.9	+	+	–
		14	84.7 ± 1.6	+++	+++	–

* Control strains.

with mycelium one week later compared to the growth on wort, and the color reaction of the substrates was less intense. Conversely, the strains *A. faginea* and *P. lycii* on the G-P medium grew faster than on the S-A medium; however, the oxidation of guaiacol and syringaldazine in this case was less intense. The addition of copper as an inducer (G-P+Cu medium) promoted the production of ligninolytic enzymes, including laccases, in *C. caperata*, *L. betulina*, *P. lycii* and *T. gibbosa* but had no effect on the production of ligninolytic enzymes in the fungi *A. faginea*, *P. avellanea*, and *P. ostreatus*. The reaction with the guaiacol and syringaldazine of fungi *C. caperata* 0677, *L. betulina* 2047, *P. ostreatus* 0432, and *T. gibbosa* 1911, as well as three control strains grown on the S-A medium, correlated with the Azure B bleaching. Note that the complete bleaching of the dye was only observed for the fungi *L. betulina*, *P. ostreatus*, and *T. gibbosa*, as well as two control strains—*T. maxima* 0275 and *T. ochracea* 093. When white-rot fungi are grown on natural substrates, including wort, they synthesize both lignin and peroxidases and various oxidases capable of generating hydrogen peroxide, which is required for manifesting the catalytic activity of peroxidase [22]. The presence of the genes encoding these enzymes was shown for *Phanerochaete chrysosporium, Peniophora gigantean, Trametes ochracea* [22, 23], *Trametes versicolor* [21], and various species of the genus *Pleurotus* [24].

Submerged cultivation and detection of the laccase gene in the genome of the studied fungi. The production of laccases in submerged cultures was observed in all tested strains, except *P. avellanea* 2009 and *X. radicata* 1795. It was shown that the composition of the medium had a significant effect on the laccase activity (Fig. 2). For example, for a number of strains, the S-A medium was optimal for laccases biosynthesis: the highest activity was exhibited by strains *A. faginea* 1998 and *S. murashkinskyi* 1963. Significant induction of laccase activity by cupric salts was observed during the cultivation of *C. caperata* 0677, *L. betulina* 2047, and *T. gibbosa* 1911 (white-rot fungi of the family Polyporaceae, which are active producers of laccases) [25]. However, the enzyme activity in *T. gibbosa* 1911 cultured on S-A and G-P+Cu media differed insignificantly (3.8 and 2.3 U/mL, respectively).

Taking into account the fact that all test strains, according to the rapid screening data, were able to oxidize syringaldazine and that laccase activity was not detected during the submerged cultivation of the *P. avellanea* 2009 and *X. radicata* 1795 strains in any of the three media, we performed experiments for detecting the laccase genes in the genomes of the studied fungi.

It is known that the amino acid sequences of all laccases described to date have four conserved regions [26]: 61-TSIHWHXFQ-69, 106-FWYHSHLSTQY-116, 394-PHPFHLHGH-402, and 449-WFLH-

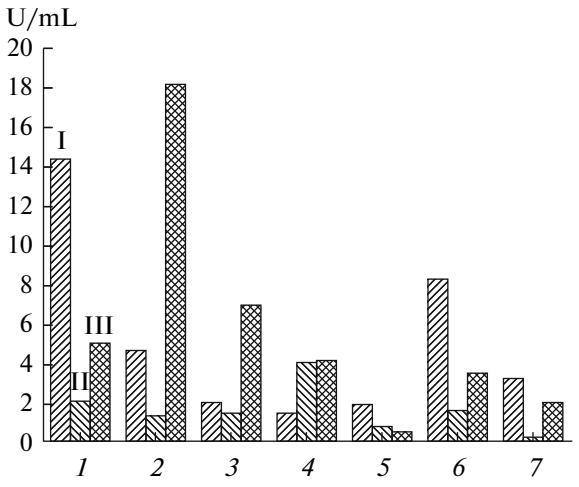

Fig. 2. Daily average laccase activity measured during submerged cultivation of fungi (*1*) *A. faginea* 1998, (*2*) *C. caperata* 0677, (*3*) *L. betulina* 2047, (*4*) *P. lycii* 2142, (*5*) *P. ostreatus* 0432, (*6*) *S. murashkinskyi* 1963, and (*7*) *T. gibbosa* 1911 on media (I) S-A, (II) G-P, and (III) G-P+Cu.

CHIDXHL-459 (the amino acid residues are enumerated according to the amino acid sequence of the laccase from *Trametes hirsuta*, PDB code 3fpx), where X designates the variable amino acid residues that are present in conserved regions. Histidine residues in these regions are responsible for the coordination of copper ions in the T1 copper site and T2/T3 copper cluster. The authors of [27] described primers for identifying laccase genes, developed for the first two regions (amino acid residues nos. 61–70 and 106–116), using which it is possible to obtain nucleotide fragments approximately 200 bp long. However, as was shown in [27], these primers cannot be used in all cases. Since laccase genes were not found in the *P. avellanea* and *X. radicata* genomes using these primers, we designed new primers. Forward primer LacA2 was selected for the second conserved region of laccase gene sequences (106-FWYHSHLSTQY-116). To construct reverse primer LacA2 Rev, we performed alignment of more than 100 basidial laccase sequences from the NCBI database, as a result of which the conserved 297-NSAILRY-303 sequence was identified. Test PCR was performed with control strains of basidiomycetes, from the culture liquids of which classic "blue" laccases were isolated and characterized: *Trametes maxima* 0275 (synonym *Cerrena maxima*), *Trametes ochracea* 093 (synonym *Coriolus zonatus*), and *Trametes hirsuta* 072. The complete nucleotide sequences of the laccase genes of these basidiomycetes were also determined [28]. PCR with the use of LacA2 For–LacA2 Rev primers yielded 750-bp amplicons for all test and control fungal genomes (Fig. 3a). The amplification specificity was verified by sequencing PCR amplification products. Since the presence of a PCR fragment, the nucleotide sequence of which corresponds to laccase, cannot guarantee that this sequence was not amplified from a pseudogene, we performed a transcriptome analysis for the presence of

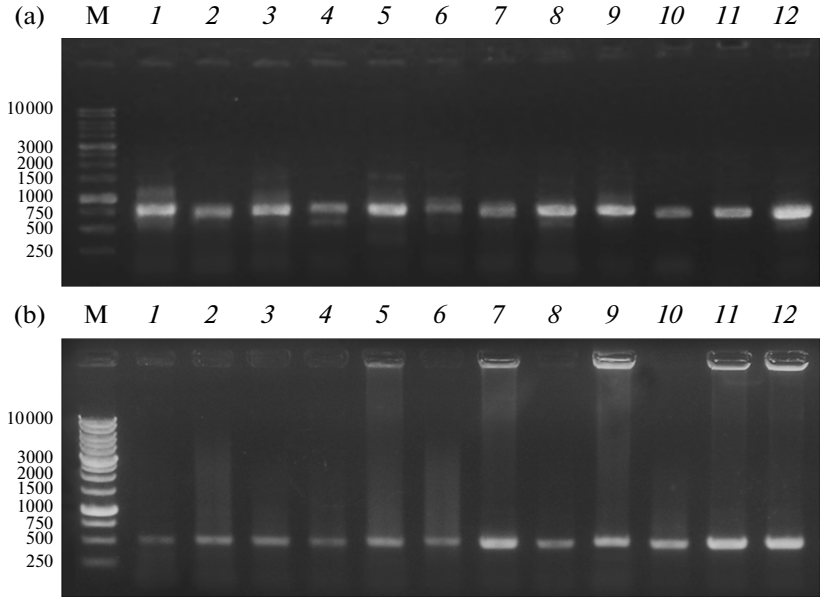

Fig. 3. Electrophoresis of amplification products obtained by PCR with primers LacA2 For and LacA2 Rev of (a) chromosomal DNA and (b) cDNA of test and control fungal strains in a 1.2% agarose gel: (*1*) *A. faginea* 1998, (*2*) *C. caperata* 0677, (*3*) *L. betulina* 2047, (*4*) *P. avellanea* 2009, (*5*) *P. ostreatus* 0432, (*6*) *P. lycii* 2142, (*7*) *S. murashkinskyi* 1963, (*8*) *T. gibbosa* 1911, (*9*) *T. hirsuta** 072, (*10*) *T. maxima** 0275, (*11*) *T. ochracea** 093, and (*12*) *X. radicata* 1795. M—DNA markers (1 kB). * Control strains.

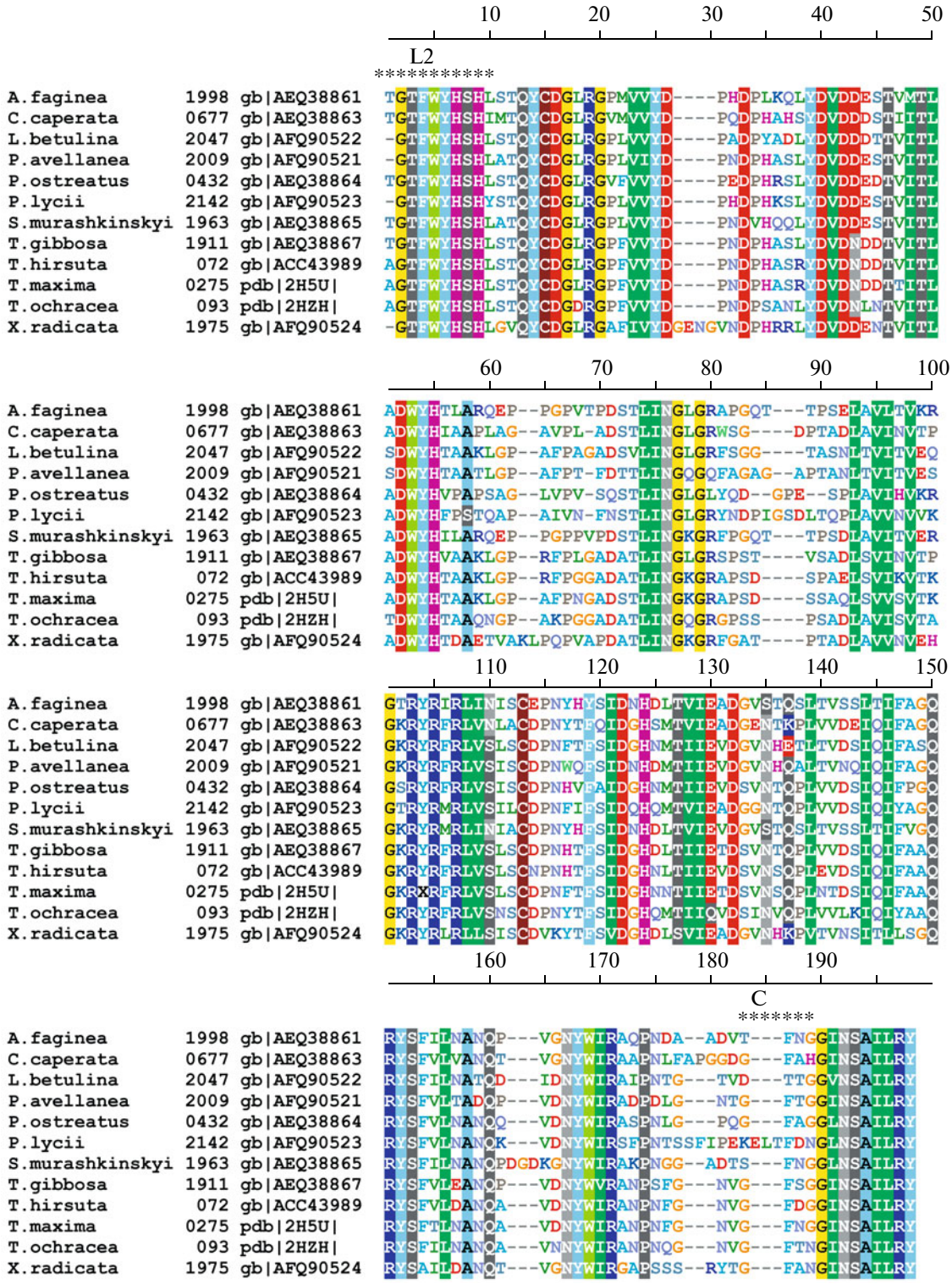

Fig. 4. Comparative analysis of the amino acid sequences of partial laccase sequences obtained using the cDNA of the studied fungi (NCBI accessions: AEQ38861, AEQ38863, AFQ90522, AFQ90521, AEQ38864, AFQ90523, AEQ38865, AEQ38867, and AFQ90524) and the amino acid sequences of the laccases of the control strains. Asterisks indicate the conserved regions.

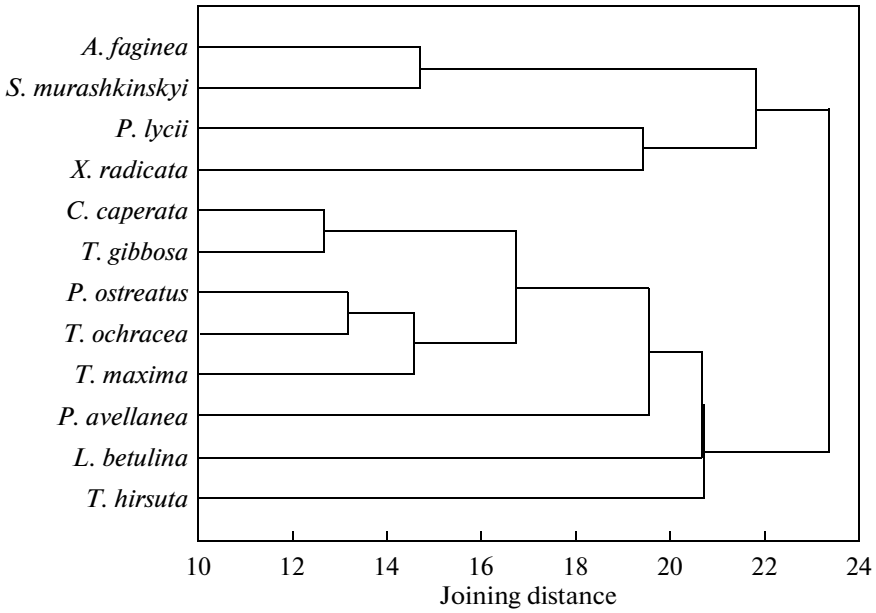

Fig. 5. Tree clustering of test and control fungal strains constructed on the basis of the ligninolytic activity determined by rapid screening, growth rate, and daily average laccase activity in a submerged culture.

using the LacA2 For and LacA2 Rev primers. PCR amplification products approximately 550 bp long were obtained for all test and control fungal genomes (Fig. 3b). Thus, expressed laccase genes were found in the genomes of all studied basidiomycetes (Fig. 4). However, to ensure the synthesis of laccases in submerged cultures of *P. avellanea* 2009 and *X. radicata* 1795, the cultivation conditions for these strains should be optimized, because the biosynthesis of laccase in different strains of white-rot fungi may vary depending on many factors, including the type and content of carbon and nitrogen sources, the presence of inducers in the medium, the phase of the biological cycle of the fungus, etc. [29].

Cluster analysis. The results of tree clustering (Fig. 5) showed that all the fungal strains studied can be divided into two major groups that live in nature on the wood of deciduous trees of varying degrees of decomposition (Table 1). An analysis of the ecology of the fungi comprising these groups showed that the first group included fungi of the families Pleurotaceae, Polyporaceae, and Phanerochaetaceae, which are the primary wound xylotrophs or inhabitants of dead, although not heavily decayed, wood. The second group was formed by members of the families Marasmiaceae, Peniophoraceae, and Steccherinaceae, which are secondary xylotrophs preferring heavily decomposed wood. A comparison of the ligninolytic activity of the studied strains and the clustering analysis results showed that the most active ligninolytic fungi belong to the first group, which allows for the primary screening of effective producers of ligninolytic enzymes among fungi belonging to different taxonomic and ecological groups.

Thus, the screening performed in this study revealed basidiomycete strains with a high ligninolytic activity and degradation potential— *Coriolopsis caperata* 0677, *Lenzites betulina* 2047, *Pleurotus ostreatus* 0432, and *Trametes gibbosa* 1911—which are promising for further use in biotechnology.

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