Lignin-Degrading Abilities of Novel Autochthonous Fungal Isolates *Trametes hirsuta* F13 and *Stereum gausapatum* F28

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SUMMARY

The aim of this research is to isolate and identify fungi with high lignin-degrading abilities that are autochthonous to southern Serbian region. Two novel fungal isolates identified as *Trametes hirsuta* F13 and *Stereum gausapatum* F28 were selected to assess their ligninolytic enzyme activities and the efficiency of lignin removal from beech wood sawdust. Obtained results show that both isolates are good sources of industrially valuable enzymes with a potential for application in various biotechnological and industrial processes. Both isolates showed laccase, manganese-dependent peroxidase, and versatile peroxidase activities, while only *S. gausapatum* F28 had lignin peroxidase activity. This is the first record of the ability of *S. gausapatum* species to produce lignin peroxidase. *T. hirsuta* F13 showed higher laccase activity than *S. gausapatum* F28, while *S. gausapatum* F28 had higher manganese peroxidase activity. Also, *T. hirsuta* F13 exhibited much higher laccase activity under submerged cultivation conditions than solid-state cultivation conditions, which is rare for fungi. This is important for industrial processes since the submerged fermentation is a dominant technique in industry. The test of the efficiency of lignin removal showed that both isolates are efficient lignin decomposers. After five weeks of incubation on beech wood sawdust, the total lignin losses were 33.84 % with *T. hirsuta* F13 and 28.8 % with *S. gausapatum* F28.

Key words: *Trametes hirsuta*, laccase, manganese peroxidase, *Stereum gausapatum*, lignin removal, white-rot fungi

INTRODUCTION

During the course of evolution, fungi have developed mechanisms to access energy incorporated in recalcitrant plant biomass in the form of polysaccharides. These mechanisms are based on the activity of various enzymes capable of breaking inter- and intrapolymer bonds of the lignocellulosic substrate, thus releasing fermentable sugars. According to the type of enzymatic activity required for a bond cleavage, lignocellulose-degrading mechanisms form two groups: hydrolytic and oxidative. Hydrolytic enzymes (cellulase and hemicellulases) break glycosidic bonds (β-1,4-, β-1,3-, β-1,2- and β-1,6-) that connect subunits of lignocellulose polysaccharides. Lignin subunits are mainly linked by ether-like or carbon-carbon bonds that cannot be hydrolyzed [1]. Its degradation occurs through oxidative processes catalyzed by fungal enzymes which include: manganese-dependent peroxidase (MnP, EC 1.11.1.13), lignin peroxidase (LiP, EC 1.11.1.14), versatile peroxidase (VP, manganese-independent peroxidase, EC 1.11.1.16) and laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) [2].

Being the most effective decomposers of lignocellulosic materials, filamentous fungi have attracted attention in the past few decades for extensive examination for applications in biorefinery and various industrial processes. The use of agricultural or other types of lignocellulosic waste for the production of biofuels or other useful fermentable products is hindered by its recalcitrance towards microorganisms and their enzymes [3]. Finding an efficient and inexpensive method for lignin removal is one of the primary objectives in the exploitation of lignocellulosic waste. Use of wood-decay fungi for lignin removal is an attractive method because it does not require additional energy and toxic chemicals, and

generates no hazardous by-products. The wood-decay fungi and their ligninolytic mechanisms are also interesting for the removal of heavy metals and toxic compounds from polluted water and soil (4–6). It has been proven that they can transform toxic organic chemicals (PCDDs, PAHs, PCBs) to less harmful or harmless forms (7–11). Fungal enzymes can also degrade recalcitrant azo dyes (which make about 70 % of all industrial dyes) and pesticides (e.g. DDT, lindane and widely used herbicide glyphosate) (12–14).

Over the last decade, the frequency of reported new wood-decomposing fungal strains has grown. Search for better lignin-degrading systems encouraged the study of fungi native to different geographical regions and adapted to different climate types. Efficient lignin-degrading mechanisms offer more ecologically and economically favourable alternative solutions for different biotechnological and/or agricultural processes (15). Besides the global impact, the use of indigenous strains has advantages at the local level as well, since it preserves autochthonous fungal flora, and is expected to remove autochthonous lignocellulosic waste more efficiently than commercial strains.

There is not much information about lignin-degrading systems of fungi that are autochthonous to Serbia. So far, there has been only data on ligninolytic abilities of few species from genera *Trametes* and *Ganoderma* (16–19) collected in the central and western territory (the area of Belgrade and Loznica, respectively).

The aim of this research is to isolate and identify novel fungi with high lignin-degrading ability autochthonous to southern parts of Serbia. The focus of this paper is on two isolates, *Trametes hirsuta* F13 and *Stereum gausapatum* F28, collected in the area of the city of Leskovac and identified according to their morphological and molecular characteristics. Evaluation of ligninolytic abilities of fungal isolates included an examination of the activities of ligninolytic enzymes and lignin removal from beech wood sawdust, which served as a model for lignocellulosic material.

MATERIALS AND METHODS

Chemicals

A local sawmill, located in the village of Strojkovce, 12 km south of the City of Leskovac, Serbia (N 42°54'10.976", E 21°55'7.658"), provided beech wood sawdust. Reagents for detection of enzyme activities were: guaiacol (99 %; ACROS Organics, Thermo Fisher Scientific, Fair Lawn, NJ, USA), Azure B (≥89 %, certified by the Biological Stain Commission; Sigma-Aldrich, Merck, St. Louis, MO, USA) and phenol red (Merck, Darmstadt, Germany). Chemicals for preparation of buffers were: tartaric acid ((+)-tartaric acid, extra pure, SLR, crystals; Fisher Chemical, Fisher Scientific, Loughborough, UK), sodium lactate (solution, extra pure, SLR; Fisher Chemical, Fisher Scientific), lactic acid (DL-lactic acid approx. 90 %; Sigma-Aldrich, Merck, Steinheim, Germany) and sodium acetate (anhydrous, laboratory reagent grade; Fisher Chemical, Fisher Scientific).

Other chemicals were: hexadecyltrimethylammonium bromide (CTAB; for molecular biology, ≥99 %) and β-mercaptoethanol, (>99 %), both from Sigma-Aldrich, Merck, Steinheim, Germany.

Isolation and maintenance of fungi

Two fungi, collected from an oak forest and orchard near the forest on a hill in the area of the city of Leskovac (in southern Serbia), were under investigation for lignocellulolytic activity. Details of the locality where they were collected are in **Table 1**. A surface sterilization technique with 70 % ethanol was used for isolation of fungal cultures from fruiting bodies. Fungi were isolated onto potato dextrose agar (PDA; preparation of one litre required: 20 g dextrose, 15 g agar and 200 g potatoes, boiled for 30 min and then filtered through two layers of cheesecloth; pH=6.2). After mycelium formation, small pieces from the edges of the actively growing fungus were transferred to a fresh agar every 3–5 days until a pure culture was obtained. Purified cultures were maintained at 30 °C on PDA and transferred to fresh agar every 2–3 weeks, to be prepared for immediate use. The stocks were prepared and stored at 4 °C on PDA slants and transferred to a fresh slant agar once every six months.

Table 1. Details of the location where the fungi were collected

Locality	Fungal isolate	
	Trametes hirsuta F13	*Stereum gausapatum* F28
Isolation source	Cherry wood branch	Oak wood branch
Host	*Prunus cerasus*	*Quercus* sp.
Locality	Area of Leskovac, Serbia	Area of Leskovac, Serbia
GPS coordinates	N 43°4'0.871" E 21°54'6.056"	N 43°4'1.831" E 21°54'6.21"
Altitude	325 m	325 m
Season	Winter	Spring
Collection date	6 January 2014	5 June 2014
Daily temperature range on the day of sample collection	12–16 °C	25–28 °C

Inoculum preparation and cultivation conditions

An agar disc of 10 mm in diameter was taken from the edge of the actively growing culture of each purified isolate, aseptically transferred to inoculum agar medium, and subsequently incubated at 30 °C, in the dark, for 7 days. Agar medium used for inoculum preparation consisted of (in g/L): NH_4NO_3 2.0, yeast extract 1.0, glucose 10.0, KH_2PO_4 1.0, $MgSO_4 \cdot 7H_2O$ 0.5, $CaCl_2 \cdot 2H_2O$ 0.01 and agar 15.0. Prior to sterilization, the pH was adjusted to 5.5. Six agar discs (10 mm in diameter) were cut from the edge of actively growing cultures and aseptically transferred to the substrate for enzyme production. Equal amount of inoculum was used for the inoculation of solid and liquid substrates. In qualitative analysis, Petri plates were inoculated with one agar disc placed in the middle.

Qualitative analysis of enzyme activity

Lignin-modifying enzyme basal medium (LBM) was prepared according to Pointing protocols (20), with NH_4NO_3 instead of ammonium tartrate. The pH was adjusted to 5.5. Lignin-modifying enzymes were assayed on LBM supplemented with 0.05 % (by mass per volume) guaiacol and 0.005 % (by mass per volume) α-naphthol for laccase activity, and 0.01 % (by mass per volume) Azure B for peroxidase activity. The guaiacol and Azure B were added prior to sterilization to the appropriate basal medium, while α-naphthol was added after sterilization, prior to use. The qualitative assay was performed in Petri dishes 90 mm in diameter.

Substrates for quantitative analysis

Screening for ligninolytic enzymes

Enzymes were produced on beech wood sawdust supplemented with a mineral medium for ligninolytic enzyme production (LMM) (21). LMM consisted of (in g/L): NH_4NO_3 0.5, KH_2PO_4 1.0, $MgSO_4·7H_2O$ 0.5, yeast extract 1.0, $CaCl_2·2H_2O$ 0.01, $CuSO_4·5H_2O$ 0.05, $MnSO_4·H_2O$ 0.03, $FeSO_4·7H_2O$ 0.05, $ZnSO_4·7H_2O$ 0.035 and $CoCl_2·6H_2O$ 0.02. The pH value was adjusted to 5.5 prior to sterilization. Solid-state fermentation (SSF) was carried out in the dark, at 30 °C, in 100-mL Erlenmeyer flasks that contained 5 g beech wood sawdust as a carbon source and 9 mL mineral medium (70 % substrate humidity). Submerged fermentation (SF) was carried out under static conditions, also in the dark at 30 °C, in 300-mL Erlenmeyer flasks loaded with 3 g sawdust and 50 mL LMM. All fermentations were performed in duplicates. Prior to inoculation, the substrates were sterilized in an autoclave for 30 min.

Testing of the moisture effect on enzyme activity

Sawdust supplemented with LMM of the same composition as that used for screening process (see the previous chapter) was used to test the effect of substrate moisture on ligninolytic enzyme activity. Two different moisture contents were used: 68 and 75 %. The activities were tested on the seventh day of incubation.

Time course of ligninolytic enzyme activity and protein production on a simple substrate

The ability of selected fungi to produce ligninolytic enzymes on a simple solid substrate (SSdS) was tested on beech wood sawdust with added dH_2O. During ten days of incubation, laccase and manganese-dependent peroxidase (MnP) activities were measured on the third, fifth, seventh and tenth day. The activities were compared to the enzyme activities obtained on beech wood sawdust supplemented with LMM (lignocellulose-mineral substrate, LMS). Total moisture content of both substrates was adjusted to 75 %.

Extraction of secreted enzymes

Enzymes produced in SSF were extracted with 50 mL of distilled water. Water was added to Erlenmeyer flasks and subsequently shaken for 30 min at 220 rpm and 30 °C. The liquid phase was separated from the solid phase by filtration through Whatman no. 1 filter paper at 4 °C and centrifugation at 4185×*g* at (25±2) °C using Z 206 A high capacity, compact centrifuge (HERMLE Labortechnik GmbH, Wehingen, Germany). SF went through the same separation process. The extracts were stored at 4 °C until use. Enzyme activity assays were performed within 24 h of the extraction.

Enzyme activity assays

Enzyme activity was calculated by the following formula:

$$EA=(\Delta A·V)/(t·\varepsilon·v·d) \qquad /1/$$

where EA is enzyme activity (U/mL), ΔA is absorbance change, V is reaction mixture volume (mL), t is incubation time (min), ε is molar absorption coefficient (L/(mmol·cm)), v is enzyme extract/filtrate volume (mL) and d is cuvette length (1 cm). Where appropriate, enzymes were properly diluted and the dilution factor was included in the formula. One unit (U) of enzyme activity was expressed as the amount of enzyme that catalyzes the oxidation of 1 µmol of substrate per min.

The absorbance was measured with UV/Visible spectrophotometer (Ultrospec 3300 pro; Amersham Biosciences Ltd, Little Chalfont, UK). All measurements were done in triplicates.

Enzyme activity per unit of dry substrate mass (U/g) was calculated by the formula:

$$EAM=(EA·V_{ex})/m_s \qquad /2/$$

where EAM is enzyme activity on solid substrate basis (U/g), EA is enzyme activity (U/mL), V_{ex} is total volume of extracted enzymes (mL), and m_s is dry mass of substrate (g).

Specific enzyme activity was calculated by the formula:

$$SEA=EA/\gamma \qquad /3/$$

where SEA is specific enzyme activity on protein basis (U/mg), EA is enzyme activity (U/mL), and γ is protein mass concentration (mg/mL).

Laccase activity assay

Oxidation of guaiacol was used to determine laccase activity. A volume of 5 mL of the reaction mixture that contained 3 mL of 10 mmol/L sodium-acetate buffer (pH=5), 1 mL of 2 mmol/L guaiacol and 1 mL of enzyme source was incubated at 30 °C for 15 min. The absorbance of oxidized guaiacol was measured at 470 nm against reference mixture that contained water instead of guaiacol. Molar absorption coefficient used for guaiacol was $\varepsilon_{470\,nm}$=6.74 L/(mmol·cm) as reported by Hosoya (22).

Manganese peroxidase activity assay

Determination of manganese peroxidase activity was based on oxidation of phenol red (23,24). A volume of 5 mL reaction mixture contained: 1 mL sodium succinate buffer (50 mmol/L, pH=4.5), 0.7 mL phenol red (0.1 mmol/L), 0.4 mL $MnSO_4$ (0.1 mmol/L), 1.0 mL sodium lactate buffer (50 mmol/L, pH=4.5), 1.0 mL gelatin (0.02 % by mass per volume) and 0.5 mL enzyme extract. The reaction started with the addition of 0.4 mL H_2O_2 (0.62 mmol/L) to the reaction mixture. Control assay without H_2O_2 and test with H_2O_2 but without Mn^{2+} were also carried out. After 5 min of incubation at (25±2) °C, the reaction was stopped with 200 µL 5 M NaOH. The absorbance was measured against reference mixture that contained inactivated enzyme (enzyme extract boiled for 15 min at 100 °C) instead of the active one, at 610 nm (molar absorption coefficient used for oxidized phenol red was $\varepsilon_{610\ nm}$=22 L/(mmol·cm)). For determination of total manganese peroxidase activity (MP), the activity of control was subtracted from the activity of reaction mixture with H_2O_2. For determination of manganese-dependent peroxidase activity (MnP), the activity of mixture without Mn^{2+} was subtracted from the activity of mixture with H_2O_2. To determine versatile peroxidase activity (VP), the activity of control was subtracted from the activity of mixture without Mn^{2+}.

Lignin peroxidase activity assay

Lignin peroxidase assay was performed according to the procedure proposed by Archibald et al. (25) and Arora and Gill (26). A volume of 2.5 mL reaction mixture contained: 1 mL sodium tartrate buffer (50 mmol/L, pH=4.5), 500 µL Azure B (32 µmol/L), 500 µL enzyme filtrate and 500 µL H_2O_2 (400 µmol/L). The reaction was initiated by the addition of H_2O_2. The absorbance decrease was measured against distilled water. The molar absorption coefficient of oxidized Azure B of $\varepsilon_{651\ nm}$=48.8 L/(mmol·cm) (25) was used for enzyme activity calculation.

Molecular identification of selected isolates

DNA extraction and PCR amplification

Fungal DNA was extracted from fresh samples. Pieces of actively growing mycelia, approx. 2.5–3 mm in diameter, were pulled out from 7-day-old fungal cultures grown on mineral agar medium of the same composition as cellulase basal medium suggested by Pointing (20) but with NH_4NO_3 instead of ammonium tartrate. They were aseptically cleaned from mineral agar and transferred to Eppendorf tubes filled with 700 µL of sterile mineral broth of the same composition as agar medium. This substrate proved to be suitable for rapid increase in the biomass of isolated fungi. Fungi were incubated for 3 days at 30 °C in the dark. Prior to DNA extraction, the samples were pelleted in a microcentrifuge (MiniSpin®, max. speed of 12 100×g; Eppendorf, Hamburg, Germany), at (25±2) °C and 4313×g for 3 min, after which the mineral medium was

discarded. This step was repeated twice, and then the samples were washed with 500 µL of 0.1× TE (Tris-EDTA) buffer (pH=8), and pelleted in the microcentrifuge (MiniSpin®) at 4313×g in order to remove the residual mineral broth. This step was also repeated twice; the first cycle lasted 5 min, the second 3 min. Prepared samples were resuspended in 2× CTAB lysis buffer prepared according to Gardes and Bruns (27). The buffer-suspended samples were frozen in the freezer at –20 °C for 15 min (instead of in dry ice) and thawed at 65 °C in heated blocs for 15 min. The cycles of freezing and thawing were repeated three times. All subsequent steps of DNA extraction were the same as in a protocol proposed by Gardes and Bruns (27).

For PCR amplification of internal transcribed spacer (ITS) region of the ribosomal DNA, a primer set ITS1/ITS4 was used (28). The extracted total DNA served as a template. The 50--µL reaction mixture contained: 1 µL of DNA template, 1 µL 10 µmol/L forward primer (ITS1: 5'-TCCGTAGGTGAACCTG-CGG-3'), 1 µL 10 µmol/L reverse primer (ITS4: 5'-TCCTCCGCT-TATTGATATGC-3'), 25 µL 2× PCR LongNova-RED Master Mix (DNA GDAŃSK, Blirt S.A., Gdańsk, Poland) and 22 µL ultrapure distilled water. The PCR amplification was performed in QB-24 Thermal Cycler (Quanta Biotech Ltd., Byfleet, UK). The temperature parameters were set as follows: 15 min at 95 °C for initial denaturation, 30 cycles of denaturation step (30 s at 95 °C), annealing step (30 s at 55 °C) and elongation step (90 s at 72 °C), and 5 min at 72 °C for final extension.

DNA sequencing and identification

The PCR products of selected fungi were examined by agarose gel electrophoresis (MultiSUB Horizontal Gel Systems, Cleaver Scientific Ltd., Rugby, UK). Ethidium bromide was used for DNA visualization. The sequencing of PCR products was performed by Macrogen sequencing service (Macrogen Inc., Amsterdam, the Netherlands). The obtained sequences were compared with the National Center for Biotechnology Information (NCBI) GenBank database (29,30). Sequences were deposited to GenBank with the accession numbers KY264754 for isolate F13 and KY264753 for isolate F28. Phylogenetic and molecular evolutionary analyses were conducted using MEGA software v. 5 (31).

Determination of protein concentration and mass fraction

Protein concentration was determined by Lowry method using bovine serum albumin as a standard (32). The amount of protein generated per unit of dry substrate mass (mass fraction) is calculated according to the formula:

$$w_m=(\gamma \cdot V_{ex})/m_s \qquad /4/$$

where w_m is protein mass fraction in dry substrate (mg/g), γ is protein concentration obtained by Lowry method (mg/mL), V_{ex} is total volume of extracted enzymes (mL), and m_s is dry mass of the substrate (g).

Determination of total solids and lignin content

Total solids in biomass were determined according to NREL/TP-510-42621 report (*33*). Acid-soluble lignin (ASL) and acid-insoluble lignin (AIL) were determined according to Laboratory analytical procedures LAP-004 and LAP-003 (*34,35*). ASL was calculated using the absorbance of hydrolysate measured at 205 nm and the molar absorption coefficient of 110 L/(g·cm).

Statistical analysis

All values are expressed as mean±standard deviation. Mean values were compared using analysis of variance (ANOVA). The impact of cultivation conditions on the fungal ability to produce adequate enzyme activity was evaluated by two-way ANOVA. Welch's ANOVA followed by Tukey's honestly significant difference (HSD) test was applied for evaluation of differences among fungi on the basis of their enzyme activity under specific cultivation conditions. Two-sample *t*-test was used for evaluation of maximal activities obtained on SSdS and LMS. Differences were considered significant at p<0.05. Statistical analyses were done using RStudio v. 1.0.136 (*36*).

RESULTS AND DISCUSSION

Identification of isolated fungi

The two isolates, F13 and F28, were subjected to identification by morphological and molecular characteristics (**Table 2**). Phylogenetic analysis was also performed (**Fig. 1**). The identities greater than 98 % with query cover higher than 95 % in the NCBI BLAST search (*37*) were considered as species-level identification.

Table 2. Data of strains/isolates with the highest similarity to F13 and F28 isolates, and obtained identities

Identified isolate	GenBank accession number	Molecular identification by BLAST algorithm		
		Fungal strain/isolate (accession number)	Query cover/%	Identity/%
Trametes hirsuta isolate F13	KY264754	*T. hirsuta* strain 394 (KC920740)	100	100
		T. hirsuta strain FCL21 (JF308949)	100	99
		T. hirsuta strain NBRC 7038 (AB733170)	100	99
		T. villosa voucher FP86588sp (JN164970)	97	99
Stereum gausapatum isolate F28	KY264753	*S. hirsutum* isolate BC (KR909200)	100	99
		S. hirsutum isolate oak16 (FN539063)	100	99
		S. hirsutum strain BS7 (KP985742)	100	99
		S. gausapatum (KF897020)	99	99
		S. gausapatum isolate st69 (FN539048)	96	99
		S. rugosum (KX218390)	90	99
		S. sanguinolentum isolate Fp-102551 (AY089730)	97	96
		S. complicatum voucher CFMR:DLL2011-039 (KJ140563)	94	96
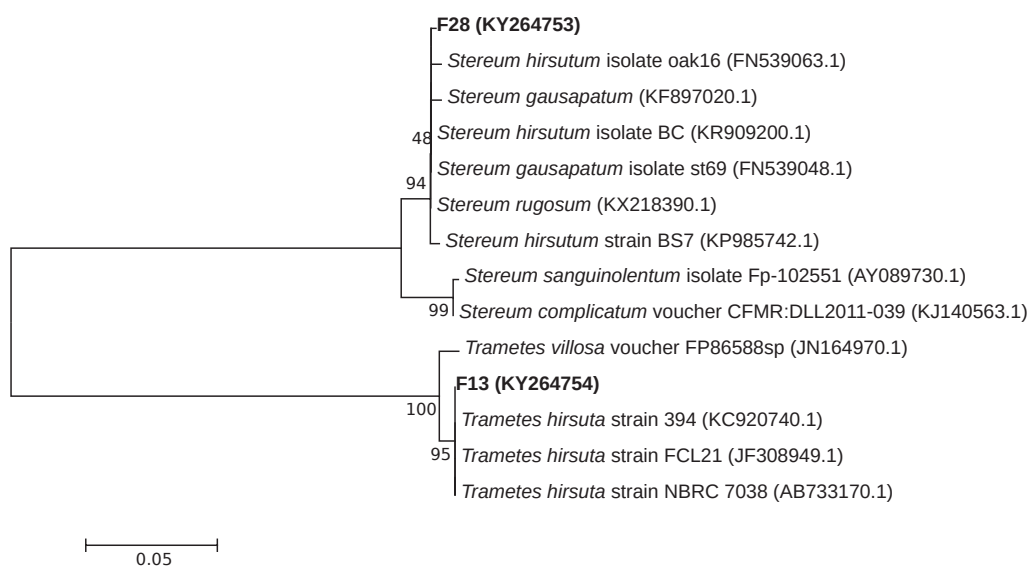
Fig. 1. Comparative phylogenetic analysis of the two selected fungi with closely related strains/isolates based on internal transcribed spacer (ITS) sequences. Parameters used for neighbor-joining tree construction: Tamura-Nei model (*31*), test of phylogeny by the bootstrap method, 1000 bootstrap replications. Accession numbers from NCBI (*30*) database of each strain/isolate are in brackets

The internal transcribed spacer (ITS) sequence of F13 showed the highest identity level with the strain *Trametes hirsuta* 394 (**Table 2**). For F28, the ITS sequence was 99 % identical with the ITS sequences of two species that belong to *Stereum hirsutum* complex: *S. hirsutum* and *S. gausapatum* with 100 and 99 % query cover, respectively. Fungi from *S. hirsutum* complex which includes *S. hirsutum*, *S. complicatum*, *S. styracifluum*, *S. subtomentosum*, *S. versicolor* and *S. gausapatum* are hard to distinguish from one another only according to the morphological characteristics. Some mycologists consider them as varieties of *S. hirsutum (38)*. A few research articles have been published in order to help in differentiating *S. gausapatum* from *S. hirsutum* (*39,40*). According to molecular, morphological and phylogenetic analyses, we concluded that the F28 isolate should be identified as *S. gausapatum*. Both isolates belong to phylum Basidiomycota, which includes the most efficient lignin decomposers in nature.

*Screening for ligninolytic enzyme activity of the fungal isolates*

Qualitative assays

Laccase activity was assayed using guaiacol and α-naphthol as reagents. Both isolates were able to oxidize these reagents; colour zones appeared within 24 h of cultivation. Isolated fungi were also tested for LiP activity by Azure B qualitative assay (*20,25*). The presence of discolouration zones under or around mycelium is a proof of LiP activity, and positive results were detected only in the case of *S. gausapatum* F28. The discolouration zones were present only under the mycelia. A percentage of the discoloured area was calculated three weeks after the inoculation. The obtained value was 10 %.

Quantitative assays

Impact of cultivation conditions (SF and SSF) on fungal ligninolytic abilities was examined by quantitative assays of enzyme activities. Both isolates were able to grow on lignocellulose substrate used in this study. Obtained activities are presented in **Fig. 2**.

Laccase activities are presented in **Fig. 2a**. Cultivation conditions had a significant impact on enzyme activities (p<0.001). Laccase activities significantly differed among isolates, both under SF cultivation conditions ($F_{1,3.1}$=3459.5, p<0.05) and under SSF cultivation conditions ($F_{1,4}$=1612.9, p<0.05). An interesting fact is that the value of (315.9±0.7) U/L recorded for the isolate F13 in SF was significantly higher than that recorded in SSF (p<0.05), although the SSF activity was also very high ((71.2±0.5) U/L). The F28 laccase activities in SSF and SF also differed significantly (p<0.05), but unlike the F13, higher activities were recorded in SSF ((18.9±0.5) U/L) than in SF ((14±1) U/L). To better explain the significance of these results, the laccase activities of isolates F13 and F28 were compared to the values of *Trametes versicolor* tested with different reagents (ABTS, 2,6-dimetoxyphenol, syringaldazine and guaiacol) according to Margot *et al.* (*41*). ABTS,
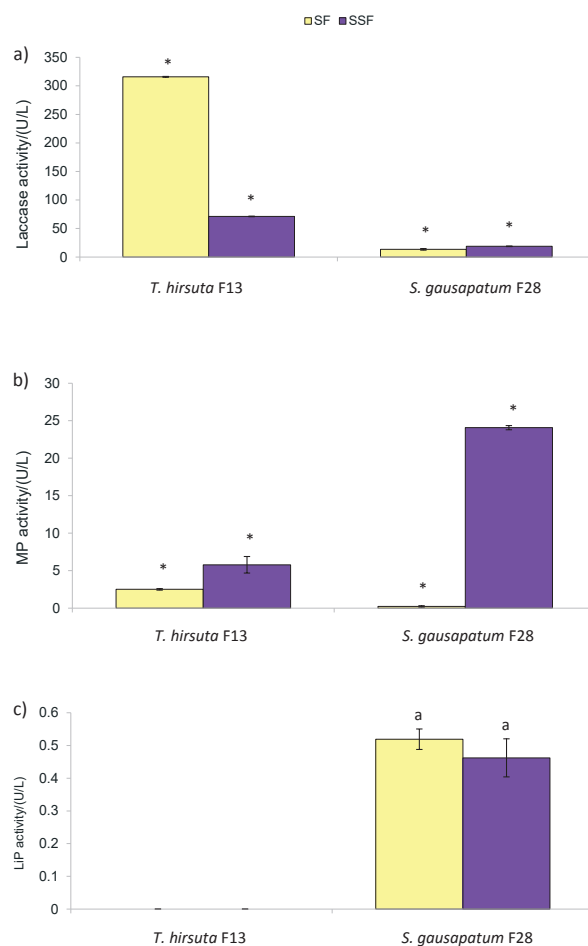
Fig. 2. Comparison of: a) laccase, b) manganese peroxidase (MP), and c) lignin peroxidase (LiP) activities obtained for fungal isolates *Trametes hirsuta* F13 and *Stereum gausapatum* F28 under different cultivation conditions: solid-state fermentation (SSF) and submerged fermentation (SF). Values are mean±standard deviation of at least three replicates. Statistically significant differences (p<0.05) are labelled with an asterisk. Letter a indicates values that did not differ significantly (p>0.05)

2,6-dimetoxyphenol, syringaldazine and guaiacol are reagents suitable for testing the laccase activity, but all of them are of different sensitivity and, accordingly, the values of the measured enzymatic activities are of a different order of magnitude. This prevents direct comparison of laccase activities tested with different reagents. Published articles usually report laccase activities that are measured using one or sometimes two of those reagents, therefore, it is not always possible to compare the obtained results with the results from the literature. The comparative study conducted by Margot *et al.* (*41*) is one of the few studies that used all four reagents for detection and measurement of laccase activity, and *T. versicolor* belongs to the group of fungi with highly active ligninolytic enzymes, which makes it suitable for comparison. Laccase activity of isolates F13 and F28 was measured using guaiacol, while the experiments with *T. hirsuta* in the literature have

mainly used ABTS or syringaldazine (*42,43*). Guaiacol is the least sensitive reagent giving much lower laccase activity values than ABTS or syringaldazine. The use of the comparative study carried out with all of the above reagents helped to conclude that both isolates, F13 and F28, possess very high laccase activity, and qualify them as laccase-producing fungi with a potential for industrial use.

MP activity of both isolates was recorded (**Fig. 2b**). Cultivation conditions had a significant impact on the MP activities (p<0.001); the obtained results showed that SSF was more suitable for MP production than SF. Measured values of MP activities also differed significantly among the isolates (in SSF cultivation $F_{1, 5.6}$=1548.2, p<0.05 and in SF $F_{1, 10}$=1753.9, p<0.05). In SSF isolate F28 showed the highest activity of (24.1±0.3) U/L, while the value for isolate F13 was (6±1) U/L. Activities measured in SF were lower than 3 U/L, therefore we concluded that none of the isolates have the potential for use in submerged biotechnological applications that require MP activity. The SSF results qualify *S. gausapatum* F28 isolate as MP-producing fungus with a potential for industrial use.

It is usually difficult to detect LiP activity in extracts from natural lignocellulosic substrates used for ligninase production by fungi (*44*). The results of LiP activity are presented in **Fig. 2c**. *S. gausapatum* F28 was able to degrade high amount of Azure B in the qualitative assay, but in the quantitative assay, the obtained activities were lower than 1 U/L. The impact of cultivation conditions was not statistically significant ($F_{1, 3.07}$=2.22, p>0.05). Because of low activities obtained in the screening process, LiP has not been studied further in this research.

Previous investigations have shown that *T. hirsuta* produces very active laccase (*45,46*), which was also noted in this research. However, the F13 did not show LiP activity. Vasina *et al.* (*42*) analyzed the production of extracellular proteins by *T. hirsuta* 072 and also found that this fungus does not produce LiP, while it does produce MP and laccase. They also reported that the laccase is a predominant enzyme in the ligninolytic system of *T. hirsuta*, which could also be applicable for the isolate F13. Differently, *S. gausapatum* F28 showed laccase, MP and LiP activities. This is the first record of LiP activity for *S. gausapatum*, while laccase and MnP activities have been reported earlier (*47*).

Effect of substrate moisture on ligninolytic enzyme activity

In further study, the focus was on laccase and manganese peroxidase activities as main ligninolytic enzymes of selected isolates. **Fig. 3** shows the effect of substrate moisture on laccase, MnP and VP activity of selected isolates. In SSF the moisture content can vary between 20 and 85 % (*48,49*), although high laccase activities of fungi are often reported with the moisture content between 60 and 80 % (*50–52*). In this research, the influence of initial moisture content on the ligninolytic activity of selected fungi was evaluated in the substrates with 68 and 75 % moisture.
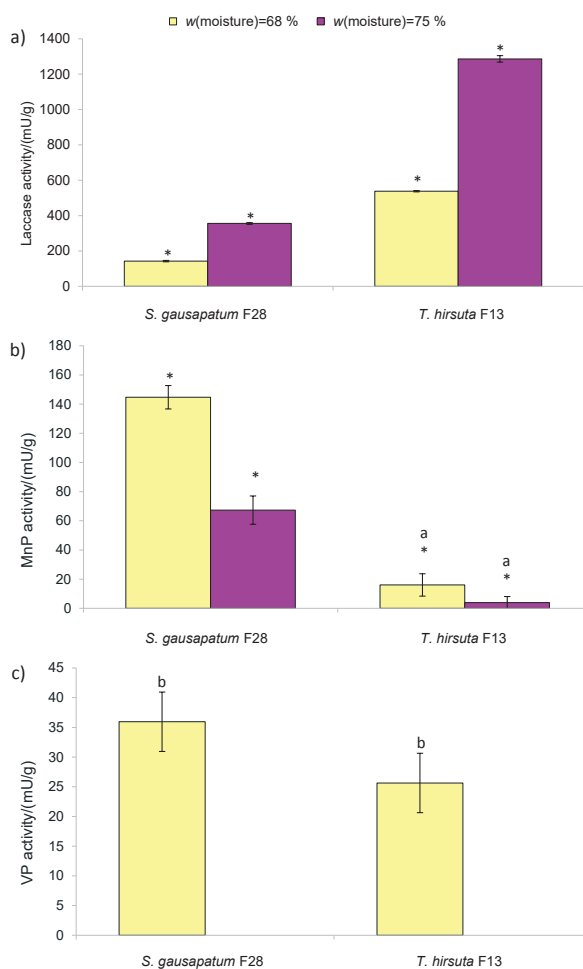
Fig. 3. Influence of substrate moisture content on ligninolytic enzyme activity of *Trametes hirsuta* isolate F13 and *Stereum gausapatum* isolate F28: a) laccase activity, b) manganese-dependent peroxidase (MnP) activity, and c) versatile peroxidase (VP) activity. Values are mean±standard deviation of at least three replicates. Activities that differed significantly (p<0.05) are marked with an asterisk. Letters a and b denote activities that did not differ significantly (p>0.05)

As shown in **Fig. 3a**, higher values of laccase activity of *T. hirsuta* F13 and *S. gausapatum* F28 were measured in substrates with 75 % moisture content than in those with 68 %. Differences were statistically significant (p<0.05). Meehnian *et al.* (*52*) analyzed the effect of the moisture content and particle size on the ligninolytic activity of *Daedalea flavida* and recorded high laccase activity in the substrate with 75 % moisture content ((4.26±0.38) U/g assayed with ABTS). *T. hirsuta* F13 and *S. gausapatum* F28 also showed very high laccase activity with 75 % moisture content, with the values of (1290±20) and (350±5) mU/g, respectively.

The higher value of substrate moisture content stimulated laccase activity, but it had an opposite or no effect on the MnP activity, depending on the fungal isolate (**Fig. 3b**). Both isolates showed higher MnP activity on the substrate with 68 % moisture content than with 75 %. Obtained differences were statistically significant for *S. gausapatum* F28 (p<0.05), while for *T. hirsuta* F13 they were not (p>0.05).

The moisture content also influenced VP activity. While the VP activities of (35±7) mU/g for *S. gausapatum* F28 and (26±3) mU/g for *T. hirsuta* F13 were observable with 68 % moisture content, with 75 % substrate moisture the VP activity was not detectable (**Fig. 3c**).

Time course of enzyme activity and protein production

The activities obtained by two selected fungi on SSdS were compared to those obtained on LMS. Production of enzymes was carried out under SSF with 75 % moisture content, which provides very high values of laccase activity, as previously shown. This part of the research was conducted in order to determine the ability of selected isolates to grow and produce ligninolytic enzymes on a simple substrate, so that could later be used in the delignification of lignocellulosic waste. Depending on the species, cultivation conditions and

incubation time, the ligninase activity may vary significantly (*24*). **Table 3** shows that the enzyme activities varied depending on the day of incubation, and the addition of minerals had a significant impact on that dependence (p<0.001).

Both isolates were able to produce very high laccase activity on SSdS. Activities significantly differed depending on the day of incubation (**Table 3**). *T. hirsuta* F13 achieved maximum laccase activities of (1324±7) mU/g on LMS and (830±5) mU/g on SSdS after ten days of incubation. The difference was significant (p<0.05), but SSdS activity was also extremely high. *S. gausapatum* F28 expressed the maximum activity on LMS on the tenth day of incubation ((355±5) mU/g), and on SSdS on the fifth day of incubation ((144.4±0.8) mU/g).

As expected, higher MnP activities were obtained with *S. gausapatum* F28 than with *T. hirsuta* F13. Activities differed significantly depending on the day of incubation (**Table 3**).

Table 3. Enzyme activity and protein production per dry substrate mass and specific activity obtained on lignocellulose-mineral substrate (LMS) and simple solid substrate (SSdS) by selected isolates

Enzyme	Isolate	Substrate	*t*(incubation)/day			
			3	5	7	10
			Activity/(mU/g of solid substrate)			
Laccase	*T. hirsuta* F13	LMS	481±1	1120±50	(1290±20)[e]	(1324±7)[e]
		SSdS	(389.2±0.8)[a]	621±1	(815±5)[d]	(830±5)[d]
	S. gausapatum F28	LMS	(137±3)[b]	252±5	(356±7)[a]	(355±5)[a]
		SSdS	(104.4±0.8)[bc]	(144.4±0.8)[b]	(74±1)[c]	(63±1)[c]
MnP	*T. hirsuta* F13	LMS	-	(6±1)[a]	-	(1.37±0.01)[a]
		SSdS	(12±1)[a]	(8±2)[a]	(8.7±0.2)[a]	(12±1)[a]
	S. gausapatum F28	LMS	(6.18±0.01)[a]	(27.8±0.5)[b]	67±9	(3.4±0.1)[a]
		SSdS	(5.8±0.5)[a]	(25±1)[bc]	39±1	(20.3±0.5)[c]
VP	*T. hirsuta* F13	LMS	6±1	-	-	-
		SSdS	-	8±2	-	-
	S. gausapatum F28	LMS	-	-	-	-
		SSdS	-	-	-	-
			w(protein)/(mg/g)			
	T. hirsuta F13	LMS	2.0±0.2	2.2±0.2	2.6±0.4	2.8±0.6
		SSdS	2.4±0.2	2.4±0.7	(4.2±0.4)*	2.7±0.3
	S. gausapatum F28	LMS	3.3±0.4	2.8±0.1	2.5±0.2	3.5±0.1
		SSdS	(3.9±0.2)*	3.2±0.2	(5.9±0.4)*	(4.2±0.2)*
			Activity/(mU/mg of protein)			
Laccase	*T. hirsuta* F13	LMS	(150±20)[a]	(410±20)[d]	510±30	(380±10)[d]
		SSdS	(99±5)[e]	190±10	(140±10)[a]	197±9
	S. gausapatum F28	LMS	(41±5)[b]	(91±4)[e]	(141±9)[a]	(101±4)[e]
		SSdS	(27±1)[c]	(45±2)[b]	(12.4±0.8)[c]	(15.0±0.7)[c]
MnP	*T. hirsuta* F13	LMS	-	(2.11±0.09)[a]	-	(0.39±0.01)[a]
		SSdS	(3.0±0.1)[a]	(2.6±0.1)[a]	(1.46±0.09)[a]	(2.9±0.1)[a]
	S. gausapatum F28	LMS	(1.9±0.2)[a]	(10.0±0.4)[b]	27±2	(0.97±0.03)[a]
		SSdS	(1.48±0.07)[a]	(7.8±0.4)[cb]	(6.5±0.4)[c]	(4.8±0.2)[ac]
VP	*T. hirsuta* F13	LMS	1.8±0.2	-	-	-
		SSdS	-	2.3±0.1	-	-
	S. gausapatum F28	LMS	-	-	-	-
		SSdS	-	-	-	-

*The measured values of protein mass fractions that are significantly different from the others (p<0.05). Differences among activity values obtained for a particular enzyme were statistically significant (p<0.05). The same letter in superscript denotes values that do not differ significantly. MnP=manganese-dependent peroxidase, VP=versatile peroxidase

Strain F28 achieved maximum activity after seven days of incubation on both substrates. Higher values were obtained on LMS ((67±9) mU/g) than on SSdS ((39±1) mU/g); differences were statistically significant (p<0.05) but the obtained values indicated the ability of *S. gausapatum* F28 to produce high MnP activity on simple lignocellulosic substrate, which can be improved by the adjustment of appropriate conditions. *T. hirsuta* F13 showed better results on SSdS, although all obtained MnP activities were rather low (**Table 3**). The VP activity was not noticeable during any of the incubation days of *S. gausapatum* F28, which could be related to the inadequate moisture content since previous tests showed its ability to produce VP on a substrate with 68 % moisture content. The VP activities of *T. hirsuta* F13 were observable on the third day of incubation on LMS ((6±1) mU/g) and on the fifth day of incubation on SSdS ((8±2) mU/g).

Actual protein mass fractions during incubation served to calculate and compare the specific enzyme activities. **Table 3** gives protein mass fractions and specific activities. Specific enzyme activity is a measure of enzyme purity. Higher activity with lower protein mass fraction means that during the enzyme production lower mass fractions of undesirable proteins are produced. In this research, cultivation on LMS gave better results. LMS gave significantly higher enzyme activities (p<0.05), while SSdS produced much higher protein mass fractions. These results showed that the addition of minerals to media can improve product quality if their purpose is for enzyme production. However, the maximal specific laccase activities of (197±9) and (45±2) mU/mg, and MnP activities of (3.0±0.1) and (7.8±0.4) mU/mg of *T. hirsuta* F13 and S. *gausapatum* F28, respectively, on SSdS confirmed that these two fungi are good source of highly active ligninolytic enzymes and that both could be used for lignin removal from lignocellulosic waste. The lignin removal was subsequently examined in this work.

Biomass reduction and lignin removal

In this research, delignification of lignocellulosic material by two selected isolates was examined on beech wood sawdust. Beech wood sawdust is a waste from wood processing and forestry industry that is widely available and cheap in Serbia. This substrate contains small particles, and its preparation does not require additional time and energy. Also, it acts as an inducer of ligninolytic enzymes such as laccase (*53*). Substrates were separately inoculated with isolates F13 and F28 and incubated for 35 days in the dark at 30 °C. A share of remaining lignin and lignocellulosic substrate determined before and after biological treatment is presented in **Fig. 4**.

T. hirsuta and *S. gausapatum* are saprophytic fungi that belong to a group of white-rot fungi. This group includes fungi able to decompose lignocellulose selectively by degrading lignin rather than holocellulose and fungi that degrade lignocellulose non-selectively by simultaneous removal of lignin and holocellulose. A selective degradation by *T. hirsuta* has already been reported (*43*). This type of lignin removal
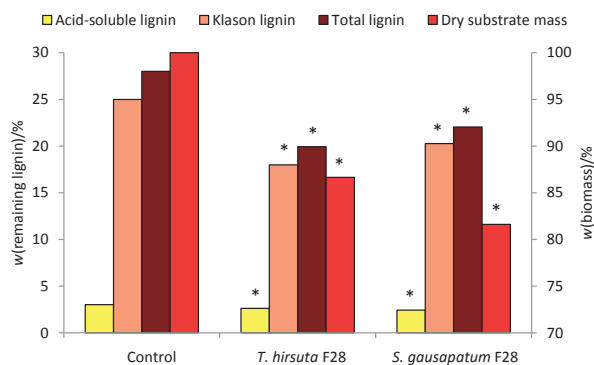
Fig. 4. Lignin removal and substrate reduction by selected fungi. The bar chart represents the percentage of remaining dry mass of the dry substrate, Klason lignin, acid-soluble lignin and total lignin mass fractions before and after pretreatment. Asterisk indicates statistically significant differences (p<0.05) between the remaining lignin (lignin mass after pretreatment) and control (initial lignin mass) and remaining lignocellulosic biomass and control. All values are calculated relative to the initial biomass

is preferable for pretreatment of lignocellulosic substrates since it preserves higher amounts of holocellulose. Ligninolytic abilities of *S. gausapatum* have not been examined before, although the studies of dead wood from which fungi were collected have shown that *S. gausapatum* causes simultaneous white rotting (*54*). As a primary colonizer of oak branches, *S. gausapatum* is expected to possess good ligninolytic abilities (*55*).

Isolates F13 and F28 reduced the amount of lignocellulosic biomass. A loss of 19 % was recorded for *T. hirsuta* F13 and 24 % for *S. gausapatum* F28. Reduction of initial biomass was statistically significant (p<0.05). Despite the fact that *S. gausapatum* F28 reduced more biomass than *T. hirsuta* F13, the difference was not statistically significant (p>0.05). As expected, both isolates were able to degrade lignin. Total lignin reduction was significant (p<0.05). Total lignin loss obtained with F13 was 33.8 and with F28 28.8 %. *T. hirsuta* F13 better degraded Klason lignin (28 % loss) than ASL (19 % loss). *S. gausapatum* F28 was able to degrade 19 % of Klason lignin and 24 % of ASL. Higher substrate reduction and lower lignin removal obtained with the isolate F28 were probably the result of higher holocellulose degradation. To analyze the efficiency of lignin removal it was necessary to compare selectivity indices. The index of selectivity was calculated as a ratio of total lignin loss to total mass loss (m_L/m_t). The higher index was obtained for *T. hirsuta* F13 (1.7) than for *S. gausapatum* F28 (1.1), which indicated higher selectivity in the decomposition of the lignocellulosic substrate. According to Liers *et al.* (*44*), m_L/m_t ratio between 1.0 and 1.9 indicates the high efficiency of lignin removal. The comparison of this with the results obtained with the selected isolates leads to a conclusion that *T. hirsuta* F13 and *S. gausapatum* F28 showed high efficiency of lignin degradation. Fukasawa *et al.* (*56*) tested decomposition of Japanese beech wood by diverse fungi, and obtained a selectivity of 1.0 for *T. hirsuta*, while we obtained much higher selectivity in this research. Lignin loss obtained here shows

that *S. gausapatum* F28 is less selective degrader than *T. hirsuta* F13, but results of ligninolytic enzyme activities, primarily on the impact of humidity on enzyme activities, indicate that the adjustment of appropriate parameters can improve selectivity.

CONCLUSIONS

We examined two novel fungal isolates able to efficiently degrade lignin. *Trametes hirsuta* F13 exhibited higher selectivity than *Stereum gausapatum* F28. The substrate moisture content, the presence of additional minerals and cultivation conditions (solid-state fermentation or submerged fermentation) had a significant influence on the type and values of fungal ligninolytic enzyme activities, especially for *S. gausapatum* F28. This implies that a correct set of parameters could improve the selectivity of lignin removal by isolate F28. In this research, we have observed lignin peroxidase activity of *S. gausapatum* for the first time as well as demonstrated its ability to efficiently remove lignin. The SSF proved to be more suitable for the production of ligninolytic enzymes due to its similarity to the natural environment of fungi. Contrary to that, we obtained significantly higher values of laccase activity with *T. hirsuta* F13 in SF than in SSF. This fungus has extremely high laccase activity in general, but the fact that SF suits it better for laccase production indicates that it is a good candidate for submerged fermentation, which is commonly used in industry. In contrast, *S. gausapatum* F28 preferred the SSF. The obtained specific activities and protein mass fractions have shown that the addition of minerals to the substrate can improve product quality. After five weeks of incubation on beech wood sawdust, lignin losses were 33.84 % with *T. hirsuta* F13 and 28.8 % with *S. gausapatum* F28. The study revealed two fungal isolates capable of efficient lignin removal and as good sources for the production of industrially valuable enzymes. They both showed the potential for applications in various biotechnological fields.

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