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RESEARCH ARTICLE

Improved Efficiency in Screening for Lignin-Modifying Peroxidases and Laccases of Basidiomycetes

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Abstract:

Background: Wood rotting white-rot and litter-decomposing basidiomycetes form a huge reservoir of oxidative enzymes, needed for applications in the pulp and paper and textile industries and for bioremediation.

Objective: The aim was (i) to achieve higher throughput in enzyme screening through miniaturization and automatization of the activity assays, and (ii) to discover fungi which produce efficient oxidoreductases for industrial purposes.

Methods: Miniaturized activity assays mostly using dyes as substrate were carried out for lignin peroxidase, versatile peroxidase, manganese peroxidase and laccase. Methods were validated and 53 species of basidiomycetes were screened for lignin modifying enzymes when cultivated in liquid mineral, soy, peptone and solid state oat husk medium.
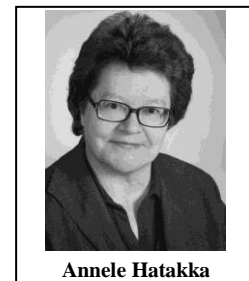
Results: Manganese peroxidases were the most common enzymes produced by 96% of the species. They typically had acidic pH optima, although *Hyphodontia* sp.*, Pleurotus pulmonarius* and *Trametes ochracea* produced enzymes highly active at pH 7. Versatile peroxidase was produced by 66% of the fungi with efficient production from *Phlebia radiata, P. pulmonarius* and *Galerina marginata*. Novel lignin peroxidase producing fungi *Cylindrobasidium evolvens* and *Daedaleopsis septentrionalis* were found among the 26% of the species showing here lignin peroxidase production. Laccase was shown in 92% of the species. Several fungi produced laccase active at pH 7, which is noteworthy because usually laccases of white-rot fungi are efficient and relevant for many industrial applications.

Conclusion: Automated screening allowed us to monitor many specific enzyme activities and extend the range of assay conditions from relatively small fungal cultivation sample volumes.

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INTRODUCTION

Lignin is a recalcitrant polymer that brings rigidity and strength to the structure of plant cell walls. White-rot fungi are able to oxidize lignin efficiently while degrading lignocellulose [1-3]. Use of lignin degrading fungi and their oxidative enzymes has been intensively studied for many potential applications such as biopulping, bleaching of chemical pulps and detoxification of waste waters or production of bio-based chemicals [2,4-6]. The possibility to use fungi and their enzymes in the pretreatment of lignocellulose before enzymatic saccharification of cellulose [7,8] and further for the production of bioethanol, has also gained much interest due to the depletion of fossil fuels and general demand for eco-friendly alternatives in sustainable economy.

Oxidoreductases involved in lignin biodegradation are peroxidases and laccases [2,3,9-12]. Peroxidases catalyze the oxidation of a wide variety of molecules utilizing H_2O_2 as the oxidant. Lignin peroxidases (LiPs, EC 1.11.1.14) are characterized by oxidation of high redox-potential aromatic compounds. Manganese peroxidases (MnPs, EC 1.11.1.13) oxidize Mn^{2+} to form Mn^{3+}-chelates, which act as diffusing oxidizers. In addition, enzymes termed as versatile peroxidases with both lignin peroxidase and manganese peroxidase type activities have first been characterized in *Pleurotus* and *Bjerkandera* species [13-15]. Versatile peroxidases (VPs, EC 1.11.1.16) have been associated with direct oxidation of high redox-potential dyes such as Reactive Black 5 (Rb5), which LiP can oxidize only in the presence of redox mediators such as veratryl alcohol [16]. Laccases (EC 1.10.3.1) are multicopper enzymes that catalyze the one-electron oxidation of phenolic compounds, with associated reduction of oxygen to water [17,18]. Compared to lignin-modifying peroxidases laccases have low redox potential that allows direct oxidation of only phenolic lignin subunits, although synthetic mediators can allow laccases to oxidize high redox-potential substrates [19].

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The discovery and engineering of oxidoreductases with improved stability, catalytic activity, and targeted substrate specificity are highly desirable for industrial biotechnology.

Pulping and bleaching are mainly performed under alkaline conditions and the waste generated is alkaline. Peroxidases and laccases of white-rot fungi usually have very acidic p*I*, and depending on the substrate in the assay, they have acidic optimum pH, e.g. laccases in the range of 2.2-6.0, with some exceptions [18]. Thus discovery of any fungal oxidoreductases active at neutral, near pH 7, or at alkaline conditions would be advantageous for further genetic engineering.

Polymeric dyes such as Poly R-478 have been successfully used in initial screening set-ups of basidiomycetes for lignin-modifying enzymes, e.g. to find efficient fungi for the degradation of polycyclic aromatic compounds on wood containing agar plates [20], or degradation patterns on wood chips [21]. Assays using polymeric dyes as substrates are easy to miniaturize and they allow handling of large number of samples. In this work two dyes, Azure B and Reactive Black 5, indicating LiP [22] and VP activities, respectively, as well as different assay conditions were used to create a representative set of data on enzyme activities of selected fungi. White-rot fungi greatly differ in their ability to degrade lignin [2,21,23] and produce different lignin-modifying oxidative enzymes [24]. Recent genome studies have revealed that there are remarkable differences in the numbers and profiles of respective genes [12,25,26] but this data does not tell which enzymes the fungi express, and thus in many cases efficient screening methodologies are needed in parallel to the information from sequenced whole genomes. In order to find novel efficient oxidoreductases with suitable properties for industrial purposes we studied culture supernatants and extracts of white-rot and litter-decomposing basidiomycetous fungi grown on several carbon sources. Four differing cultivation media were used to increase the expression profiles of enzymes. To further ensure the discovery of all produced enzymes, we assayed enzyme activities also at neutral pH in addition to the conventional acidic conditions. We also miniaturized and optimized a pattern of enzyme activity assays in order to allow higher throughput in screening and use of pipetting robots, and thus to improve the efficiency of screening and reliability of the data created. A large variety of new fungal species and strains for the production of lignin-modifying peroxidases and laccases with large pH activity range and high industrial potential was described.

MATERIALS AND METHODS

Fungal Strains

The 53 fungal strains (Table **1**) representing different taxonomical positions were obtained from the Fungal Biotechnology Culture Collection (FBCC), University of Helsinki, Finland. The fungi are grouped according to their order and family [26-28]. The selection criteria for part of the chosen fungal strains were good lignin degrading ability when grown on spruce wood blocks and rapid growth on different agar plate tests [21]. The well-known white-rot fungi *Phanerochaete chrysosporium* ME446 (ATCC4541), *Ceriporiopsis subvermispora* CZ-3 (ATCC96608) and *Phlebia radiata* 79 (ATCC64658) were used as references.

Fungi belonging to the order Agaricales are mostly litter-decomposing fungi. Table **1** also shows those fungi the genomes of which are sequenced and the data publicly available [12,29-33]. The fungi were maintained on 2% (w/v) malt extract (Biokar) agar plates.

Culture Conditions

We used three liquid culture media and one solid medium suitable for lignin-modifying enzyme production and especially for peroxidase production. The media were a) low nitrogen (2 mM) asparagine-dimethyl succinate-medium (M), containing 0.5% (w/v) glucose, pH 4.5 [23], b) peptone medium (P) with yeast extract 0.2% (w/v), peptone 0.5% (w/v), glucose 2% (w/v), 7 mM KH_2PO_4, 2 mM $MgSO_4$ and 100 µM $MnSO_4$, pH 5 (modified from [34]), c) soy medium (S) with 2% (w/v) soy briquettes (Mildola Ltd, Kirkkonummi, Finland), water, pH adjusted to 5, and d) solid oat husk (Rapion Tuote Inc., Finland) medium (O) with water supplementation [35]. For the production of inocula fungi were cultivated in 2% (w/v) malt extract medium and the mycelium with the medium was homogenized in a Waring blender four times 10 sec in 30 sec intervals. For enzyme production, 100 ml flasks with 15 ml liquid medium per flask were inoculated with 3% (v/v) of homogenized mycelium and the fungi were cultivated stationary at 25ºC for 7, 14 and 21 days.

Liquid cultures were filtered through Miracloth (Millipore, Germany). Samples from solid state cultivations were extracted in a ratio liquid: solids = 6:1 (4 g milled, to pass 2 mm sieve, oat husks humified by 8 ml water and extracted by 16 ml 0.05 M sodium phosphate buffer, pH 6.5) as described previously [35]. The culture filtrates and extracts were centrifuged for 2 min 16,000*g and 50 µl supernatants were used for enzyme assays. In order to enhance the specificity of the laccase assay, culture supernatants were treated by catalase to remove excess H_2O_2, essential for peroxidase function. Catalase (Sigma) 30 U/ml was mixed with culture supernatants and incubated at room temperature for 15 min. This treatment was considered efficient enough since according to the manufacturer 1 U of this catalase decomposes 1.0 µmol of H_2O_2 per min, while wood-rotting fungi produce 0.5 to 2.0 µM H_2O_2 [36]. After subsequent centrifugation 50 µl of supernatant was used for enzyme screening.

Enzyme Activity Assays

Microplate Assay

Laccase, lignin peroxidase (LiP), manganese peroxidase (MnP), and Mn-dependent (VP-Mn) and Mn-independent versatile peroxidase (VP-Ind) activities were determined from fungal culture supernatants and extracts. For the optimization of the miniaturized assays in 96-well plates commercial *Trametes versicolor* laccase from Sigma-Aldrich, *Bjerkandera adusta* versatile peroxidase (VP) from Jenabios (Germany) and *Phlebia* sp. (formerly *Nematoloma frowardii,* see [37]) MnP from Jenabios were used. Supernatants (50 µl) were manually pipetted to flat bottom 96-well plates (Nunc, USA), except for the assay of MnP where UV transparent 96-well plates (Falcon, USA) were used.

Biomek FX Automated liquid handling robot (Beckman Coulter) was used to transfer 150 µl buffer to peroxidase

Table 1.   **Fungal strains selected for screening, their origin of isolation, taxonomical position and lignin modifying enzyme activities detected in this study during three week cultivation.**

Fungus and Strain	FBCC [a]	Origin of Isolation	Order [b]	Family Name	LME's Detected
Exidia saccharina 343	142	Viitasaari, Finland	Aur	Auriculariaceae	MnP, Lacc
Onnia leporina PO119[d]	188	Sodankylä, Finland	Hym	Hymenochaetaceae	VP-Ind
Phellinus viticola T24[d]	694	Sodankylä, Finland	Hym	Hymenochaetaceae	MnP, Lacc
Tubulicrinis accedens T226i[d]	920	Evo, Finland	Hym	Hymenochaetaceae	MnP, Lacc, VP-Mn
Fibricium rude PO140i[d]	206	Muonio, Finland	Hym	Incertae sedis	MnP, Lacc
Trichaptum fuscoviolaceum T179	840	Evo, Finland	Hym	Incertae sedis	MnP, Lacc, VP-Ind
Trichaptum laricinum T30	699	Luosto, Finland	Hym	Incertae sedis	MnP, Lacc, VP-Ind
Basidioradulum radula T191i	873	Lammi, Finland	Hym	Schizoporaceae	MnP, Lacc, VP-Mn
Hyphodontia sp. T165	822	Juupajoki, Finland	Hym	Schizoporaceae	MnP, Lacc, LiP, VP-Mn
Resinicium bicolor T238i	938	Lammi, Finland	Hym	Incertae sedis	MnP, Lacc, LiP, VP-Mn
Resinicium furfuraceum PO175i[d]	228	Juupajoki, Finland	Hym	Incertae sedis	MnP, Lacc
Climacocystis borealis T261[d,f]	963	Kolari, Finland	Pol	Fomitopsidaceae	MnP, Lacc
Ischnoderma benzoinum 108	62	Joutsa, Finland	Pol	Fomitopsidaceae	Lacc, VP-Ind, VP-Mn
Physisporinus rivulosus T241i[d,f]	939	Lammi, Finland	Pol	Incertae sedis	MnP, Lacc
Physisporinus vitreus H	450	Hamburg, Germany	Pol	Meripilaceae	MnP, Lacc, VP-Mn
Bjerkandera sp. BOS55	395	Unknown	Pol	Meruliaceae	MnP, LiP, VP-Ind
Gelatoporia pannocincta PO115	185	Sodankylä, Finland	Pol	Meruliaceae	MnP, Lacc, VP-Mn
Irpex lacteus CCBAS	1012	Unknown	Pol	Meruliaceae	MnP, VP-Mn
Merulius tremellosus T186i	857	Evo, Finland	Pol	Meruliaceae	MnP, Lacc, VP-Ind
Phlebia centrifuga PO127[d]	195	Sodankylä, Finland	Pol	Meruliaceae	MnP, Lacc, VP-Mn
Phlebia radiata 79[c]	43	Vantaa, Finland	Pol	Meruliaceae	MnP, Lacc, LiP, VP-Ind, VP-Mn
Radulodon erikssonii T84	752	Lammi, Finland	Pol	Meruliaceae	MnP, Lacc
Ceriporiopsis subvermispora CZ-3[c,d,f]	314	Port Townsend, USA	Pol	Phanerochaetaceae	MnP, Lacc, VP-Ind, VP-Mn
Climacodon septentrionalis T99	760	Lammi, Finland	Pol	Phanerochaetaceae	MnP, Lacc, LiP, VP-Mn
Phanerochaete chrysosporium ME446[c,d,f]	280	Waterville, USA	Pol	Phanerochaetaceae	MnP, LiP, VP-Ind
Phanerochaete sordida 37	21	Perniö, Finland	Pol	Phanerochaetaceae	MnP, Lacc, LiP
Phanerochaete velutina 244i[d]	941	Kolari, Finland	Pol	Phanerochaetaceae	MnP, Lacc, VP-Ind, VP-Mn
Phlebiopsis gigantea T60iA[f]	730	Sodankylä, Finland	Pol	Phanerochaetaceae	MnP, Lacc, VP-Mn
Cerrena unicolor PM170798[f]	387	Eura, Finland	Pol	Polyporaceae	MnP, Lacc, VP-Ind, VP-Mn
Daedaleopsis septentrionalis T20	690	Sodankylä, Finland	Pol	Polyporaceae	MnP, Lacc, LiP, VP-Ind, VP-Mn
Dichomitus squalens PO114[f]	184	Sodankylä, Finland	Pol	Polyporaceae	MnP, Lacc
Diplomitoporus crustulinus T56	721	Sodankylä, Finland	Pol	Polyporaceae	MnP, Lacc, LiP
Haploporus odorus T154	804	Juupajoki, Finland	Pol	Polyporaceae	MnP, Lacc, VP-Ind
Polyporus brumalis 71[f]	40	Perniö, Finland	Pol	Polyporaceae	MnP, Lacc
Pycnoporus cinnabarinus 115 [f]	68	Joutsa, Finland	Pol	Polyporaceae	MnP, Lacc, VP-Ind, VP-Mn
Skeletocutis stellae T147	796	Lammi, Finland	Pol	Polyporaceae	MnP, Lacc

Table 1. contd….

Fungus and Strain	FBCC [a]	Origin of Isolation	Order[b]	Family Name	LME's Detected
Trametes ochracea T178	838	Sodankylä, Finland	Pol	Polyporaceae	MnP, Lacc, LiP, VP-Ind, VP-Mn
Trametes pubescens T65iB	735	Sodankylä, Finland	Pol	Polyporaceae	MnP, Lacc, VP-Ind, VP-Mn
Trametes velutina K169	592	Ylläs, Finland	Pol	Polyporaceae	MnP, Lacc, LiP
Trametes versicolor K120a1[f]	563	Gifhorn, Germany	Pol	Polyporaceae	MnP, Lacc, VP-Ind, VP-Mn
Stereum sanguinolentum SS1	1146	Oulu, Finland	Rus	Stereaceae	MnP, Lacc
Galerina marginata K96[f]	1185	Hyytiälä, Finland	Aga	Cortinariaceae	MnP, Lacc, VP-Mn
Mycena galericulata K175	598	Ylläs, Finland	Aga	Mycenaceae	MnP, Lacc VP-Mn
Cylindrobasidium evolvens 58	34	Perniö, Finland	Aga	Physalacriaceae	MnP, Lacc, LiP, VP-Ind, VP-Mn
Flammulina velutipes K158[f]	583	Jaala, Finland	Aga	Physalacriaceae	MnP, Lacc
Pleurotus ostreatus DSM11191[c,f]		Unknown	Aga	Pleurotaceae	MnP, Lacc, LiP, VP-Ind, VP-Mn
Pleurotus pulmonarius K42	517	Sodankylä, Finland	Aga	Pleurotaceae	MnP, Lacc, VP-Ind, VP-Mn
Agrocybe praecox TM70.84	476	Unknown	Aga	Strophariaceae	MnP, Lacc
Gymnopilus luteofolius X9	466	Unknown	Aga	Strophariaceae	MnP, Lacc, VP-Mn
Gymnopilus penetrans HAM1	1010	Hamina, Finland	Aga	Strophariaceae	MnP, Lacc
Kuehneromyces mutabilis K22	508	Jaala, Finland	Aga	Strophariaceae	MnP, Lacc, VP-Mn
Stropharia coronilla Stock1	480	Unknown	Aga	Strophariaceae	MnP, Lacc
Hygrophoropsis aurantiaca K192	616	Nastola, Finland	Bol	Hygrophoropsidaceae	MnP, Lacc, VP-Ind, VP-Mn

[a]Collection code for fungal strains deposited in Fungal Biotechnology Culture Collection.
[b]Order name; Aga, Agaricales; Aur, Auriculariales; Bol, Boletales; Hym, Hymenochaetales; Pol, Polyporales, Rus, Russulales
[c]*C. subvermispora* ATCC96608, *P. chrysosporium* ATCC4541, *P. radiata* ATCC64658, *P. ostreatus* ATCC38538.
[d]Selective removal of lignin in spruce wood block test [21]
[f]Genome sequence published from same species and/or the same strain [12, 30-33] or available at Joint Genome Institute webpages [30]. Synonym for *P. rivulosus* is *Obba rivulosa* T241i.

assay plate wells and 190 μl buffer to laccase assay wells. Assays were performed in 100 mM sodium tartrate buffer at pH 3, pH 5 and pH 7. Subsequently 40 μl of Azure B (Sigma, 0.125 mM), Reactive Black 5 (Aldrich, 0.125 mM) and MnSO$_4$ (Riedel-de Haën, 3.1 mM) were added to LiP, VP and MnP assay wells, respectively. Mn-dependent versatile peroxidases (VP-Mn) were measured with MnSO$_4$ (0.625 mM) and Mn-independent (VP-Ind) assays with EDTA (Fluka, 0.625 mM) and without any addition of Mn, both using Reactive Black 5 (Rb5) as substrate. Enzyme activities were measured spectrophotometrically at 25°C with Varioskan plate reader (Labsystems, Finland) equipped with a dispensing unit. Peroxidase reactions were started with 10 μl 2.5 mM H$_2$O$_2$ and laccase reactions with 10 μl 25 mM 2,6-dimethoxyphenol (Aldrich). Lignin peroxidase (LiP) activity was determined by measuring the absorbance change at 647 nm [22]. VP-Mn and VP-Ind activities were determined by measuring absorbance change at 598 nm [38]. MnP activity was determined by measuring absorbance change at 270 nm [39]. Laccase activity was determined spectrophotometrically at 476 nm by following the oxidation of 2,6-dimethoxyphenol as described previously [40].

Cuvette Assay

Enzyme activities in test tube scale and measurements in spectrophotometric cuvette were determined by the same type of protocol, except all volumes were four times larger than in microplate assay and hence total reaction volume was 1000 μl instead of 250 μl. Activities were measured with UV-1700 Pharma Spec Spectrophotometer (Shimadzu, Japan).

Optimization of Miniaturized Screening Assays

Commercial *T. versicolor* laccase, *B. adusta* VP and *N. frowardii* MnP were positive references for the respective enzyme activity assays. In addition, all the 96-well plates contained negative references of each four culture media without fungus.

Quality of the assays was estimated by Z' factor [41],

$$Z' = 1 - (3\sigma_S + 3\sigma_B) / |(\mu_S - \mu_B)|$$

where σ_S and σ_B are the standard deviation of the signal and background and μ_S and μ_B are the mean values for the signal and background. Z' value above 0.5 ensures wide separation window between signal and background, and hence is an indicator of excellent assay quality. Further, excellent assay quality allows quantitative analysis of positive values and reliable separation from the negatives. Assay performance was evaluated during optimization and validation of assay conditions as well as during screening.

RESULTS

Validation of Miniaturized Enzyme Assays

The first aim to design, optimize and validate a pattern of miniaturized oxidoreductase assays to improve efficiency, coverage, in this case a wide pH range, and throughput in screening, was approached by comparing the enzyme activities obtained in conventional cuvette (test tube scale) assay to the activities obtained by 96-well microplate assay (Table **2**). Based on calculated Z' factors for each assay, we optimized the assay conditions with the reference enzymes *T. versicolor* laccase, *Phlebia* sp. MnP and *B. adusta* VP. In each case the optimized oxidoreductase assays gave Z' values above 0.5, which is a limit for an excellent assay. In most cases the values were around 0.8, only in the case of MnP somewhat lower values were obtained. The results were considered acceptable for a quantitative analysis of the data.

Automatic Screening of Lignin Peroxidase and Versatile Peroxidase Activities by Dye Decolorization Assays

About 72% (38 out of 53) of the species produced VP or LiP type activities (Figs. **1** and **2**, Tables **1** and **3**). New LiP producing fungi, *Daedaleopsis septentrionalis* T20 and *Cylindrobasidium evolvens* 58, were found among the 13 species (26%) producing LiP but in general LiP type activity was scarce. Only in 1% of the culture liquids or extracts LiP activity was detected and only at pH 3 (Table **3**). *Phlebia radiata* 79 produced LiP in both mineral and soy medium whereas the other species could produce LiP only in one culture condition, either in mineral, soy or oat husks but not in several media. The most efficient LiP producers belonged to the genera *Phlebia, Trametes* and *Daedaleopsis.*

VP activities as determined by Rb5 decolorization, were detected in 35 (66%) of the species studied (Table **1**, Fig. **2**).

Table 2.   Comparison of laccase, manganese peroxidase and versatile peroxidase activities in cuvette and microplate assays. Values are means of 12 (96-well plate) and 4 (cuvette) replicates with standard deviation. Z'-factors are means of six different experiments performed on separate days on microplates.

Enzyme	Assay	pH	Activity (µkat/L)		Z' Factor Microplate
			Cuvette	**Microplate**	
Lacc (*Trametes versicolor*)	Laccase	5.0	1.3 ± 0.0	1.3 ± 0.0	0.8 ± 0.1
MnP (*Phlebia* sp. *Nf*b19)	MnP	5.0	7.8 ± 0.2	6.7 ± 0.8	0.6 ± 0.2
VP (*Bjerkandera adusta*)	LiP	3.0	0.05 ± 0.00	0.05 ± 0.00	0.8 ± 0.1
	VP-Mn	3.0	0.15 ± 0.00	0.11 ± 0.01	0.8 ± 0.2
	VP-Ind	3.0	0.23 ± 0.01	0.15 ± 0.01	0.8 ± 0.1

Table 3.   Share of lignin-modifying enzyme (LME) producing species (%) out of 53 fungi studied and distribution of activities detected (%) in four culture conditions and three buffer pH's out of 1908 variables for each enzyme assay.

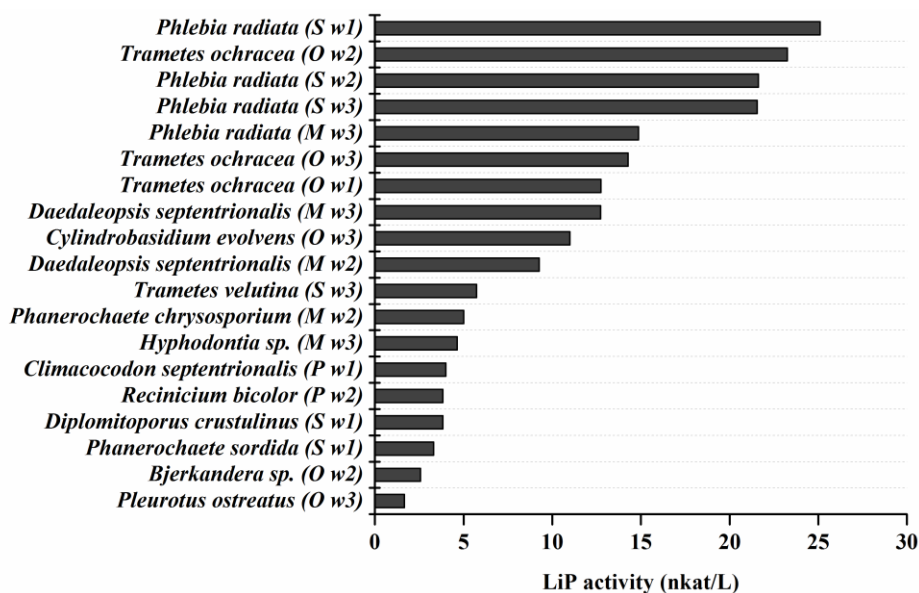| LME	Species	pH	Mineral	Peptone	Soy	Oat Husks	All Media
MnP	96	3	11	18	25	14	17
		5	36	50	53	28	42
		7	30	35	33	22	30
		All pH's	26	35	37	21	30
VP-Mn	53	3	8	9	13	11	10
		All pH's	3	3	4	4	3
VP-Ind	40	3	8	1	12	8	7
		All pH's	3	0	4	3	2
LiP	25	3	3	1	4	4	3
		All pH's	1	0	1	1	1
Lacc	92	3	20	28	63	63	31
		5	16	26	52	58	31
		7	5	27	33	55	22
		All pH's	14	27	49	59	37

Fig. (1). Lignin peroxidase activities measured by the decolourization of Azure B dye at pH 3. Culture supernatant from mineral (M), peptone (P) or soy (S) liquid media or extract of cultivation on oat husks (O) and culture duration in weeks (w1, w2, w3) is given in parenthesis. Values are means of triplicate measurements.
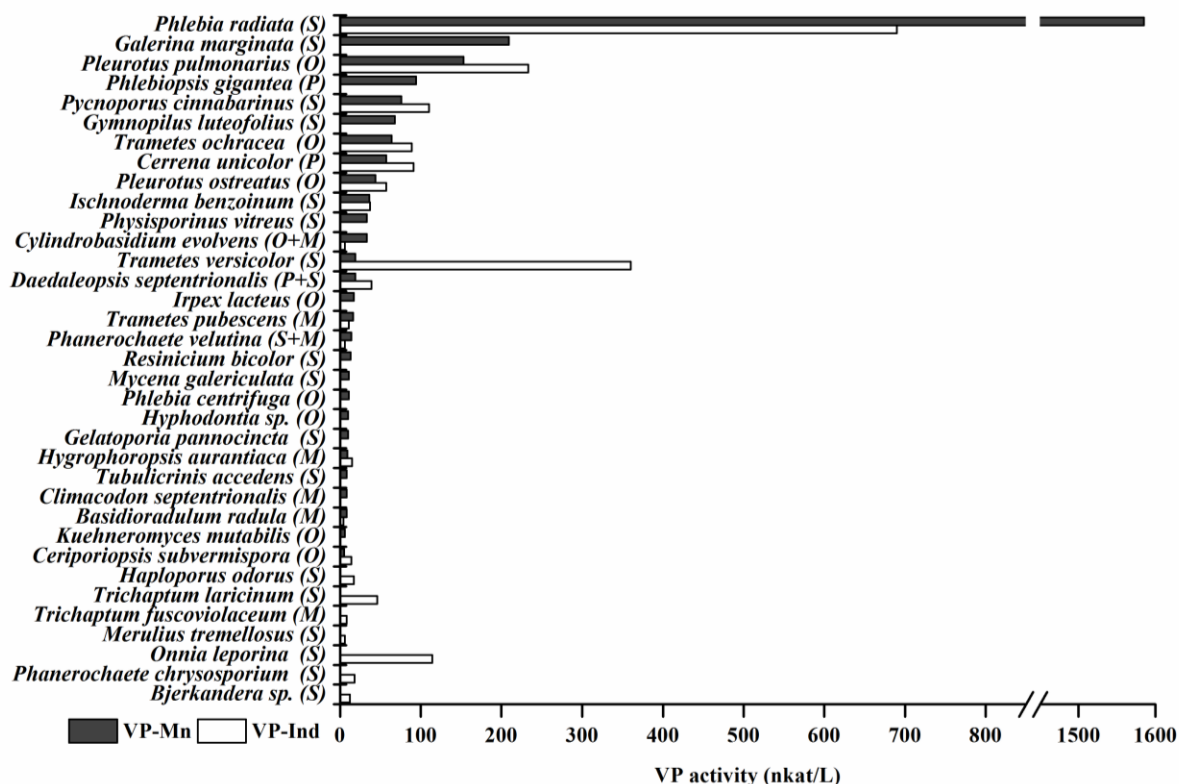

Fig. (2). Versatile peroxidase activities VP-Mn (black bars) and VP-Ind (white bars) of fungal culture supernatants from mineral (M), peptone (P) or soy (S) liquid media or extract of cultivation on oat husks (O) is given in parenthesis. VP activities were measured by the decolorization of Reactive Black 5 dye with Mn (black bars) or EDTA (white bars) at pH 3. Values are means of triplicate measurements.

Similarly to the LiP screening, VP activities were found only at pH 3. VP-Mn and VP-Ind activities were produced by 26% of the species simultaneously, and in many cases Mn-independent activities were slightly higher than Mn-dependent activities. Even so, there were more fungi producing merely VP-Mn-activities (51%) than fungi producing VP-Ind activities (40%). The highest VP producers, both VP-Mn and VP-Ind, were *Phlebia radiata* 79, *Galerina marginata* K96 and *Pleurotus pulmonarius* K42.

Automatic Screening of Manganese Peroxidase and Laccase Activities

Our results show that MnP was the most common lignin-modifying peroxidase produced by lignin-degrading and litter-decomposing fungi (Table **3**, Fig. **S1** and Fig. **S2**). Almost all tested fungi (96%) except *Onnia leporina* PO119 and *Ischnoderma benzoinum* 108, produced MnPs and MnP activities were detected in 30% of all screening samples and assay conditions, including four media, three harvesting times (1, 2 and 3 weeks) and three assay pHs (pH 3, 5 and 7). High MnP activities were produced mostly on oat husks, and also soy and peptone media supported well the production of MnPs. The good MnP producers belonged to the genera *Agrocybe, Cerrena, Climacocystis, Climacodon, Daedaleopsis, Galerina, Gymnopilus, Haploporus, Kuehneromyces, Phanerochaete, Phlebia, Pholiota, Physisporinus, Pycnoporus, Recinicium* and *Trametes*.

Fungi showing their maximal MnP activity at pH 7 were *Daedaleopsis septentrionalis* T20, *Climacocystis borealis* T261, and *Hyphodontia* sp. T165 (Fig. **S1**). Fungi showing the highest MnP activities at pH 7 when they were cultivated on oat husks were *Phanerochaete velutina* 244i, *P. pulmonarius* K42, and *T. ochracea* T178. On peptone medium the highest neutral MnP activity, i.e. the activity assayed at pH 7, was obtained with *C. borealis* T261.

Laccase activity was produced by 92% of the species studied and 37% of all culture samples in all assay conditions were laccase positive. The highest laccase activities were produced on soy and oat husk media, and were measured at pH 5. The most efficient laccase producers at pH 5 were *Cerrena unicolor* PM170798, *Hygrophoropsis aurantiaca* K192, *Kuehneromyces mutabilis* K22, *Physisporinus vitreus* H, *Agrocybe praecox* TM70.84, *Trametes ochracea*, T178, *Phlebia radiata* 79 and *Climacodon septentrionalis* T99 (Fig. **S2**). Laccases active at pH 7 were produced efficiently by fungi belonging to the genera *Physisporinus, Kuehneromyces, Cerrena, Hygrophoropsis, Agrocybe* and *Pleurotus*. Under our culture conditions *O. leporina* PO119, *Bjerkandera sp.* BOS55, *Irpex lacteus* CCBAS and *Phanerochaete chrysosporium* ME446 did not produce laccase. Under very acidic assay conditions (pH 3) the highest laccase activities were found in soy or oat husk medium. Best producers of laccases active at low pH were *K. mutabilis* K22, *C. unicolor* PM170798, *Dichomitus squalens* PO114, *Physisporinus rivulosus* T241i, *Agrocybe praecox* TM70.84, *H. aurantiaca* K192 and *Ceriporiopsis subvermispora* CZ-3.

DISCUSSION

The selection criterion for part of the 53 fungi was good performance exhibited by high lignin degrading ability, in our previous screening on spruce (*Picea abies*) wood block test [21]. In addition some litter-decomposing fungi as well as reference fungi known to be efficient producers of lignin-modifying enzymes were selected to the study. Because we studied already pre-screened fungi our results cannot be considered as a general overview or analysis of a random collection of wood-rotting basidiomycetes. White-rot basidiomycetes also prefer hardwood in nature, not softwood

such as spruce. Keeping these precautions in mind, our study gave very valuable information on the distribution and characteristics of various enzyme activities, which may well reflect the situation among white-rot and litter-decomposing fungi in general. The lignin-modifying enzymes detected in the present study are summarized in Table **1**.

Production of Lignin-modifying Peroxidases

Azure B (AzB) or Reactive Black 5 (Rb5) bleaching activities denoting LiP or VP activities, respectively, were characteristic for a rather few species and culture conditions. Different culture and extract samples assayed at pH 3, LiP activity was detected in 3%, VP-Ind activity in 7% and VP-Mn activity in 10%. Ability to bleach either both or one of these dyes were noted for 35 species, such as those in the genera *Cerrena, Phlebia, Pleurotus, Phanerochaete* and *Trametes*. However, less than ten species were excellent producers of these dye decolorizing activities. Ability to bleach Rb5 in the presence of Mn (Mn-dependent decolorization, VP-Mn) was much more common and the order of the most active fungi was different. In general VP production was common to all major orders studied. Two thirds of the species studied belonging to Agaricales, Polyporales or Hymenochaetales produced VP. Litter-decomposing species from the order Agaricales showed solely VP-Mn activity without any VP-Ind activities. Soy medium also in this case supported a high expression of Rb5 bleaching activity. One of the efficient producer of VP activity was *Cerrena unicolor* PM170798, which is known as a very efficient producer of laccase [43], like many other *C. unicolor* strains [44].

Both *P. chrysosporium* ME446 and *P. radiata* 79 bleached Rb5 indicating VP activity. However, in *P. chrysosporium* no VP gene model has been found [26], and the production of VP in *P. radiata* 79 has not been reported. It is possible that other class II peroxidase(s), either MnP or LiP, of these fungi bleached Rb5 dye, and the assay was not specific enough to separate these activities. Among lignin-modifying peroxidases, LiP and VP can directly oxidize the benzene rings of lignin [4,15]. By definition, LiPs are characterized by the oxidation of lignin on the enzyme protein surface using an exposed tryptophanyl radical (exceptionally a tyrosyl radical) by a long-range electron transfer to the activated cofactor [4,15]. VP proteins are defined by the presence of both the catalytic tryptophan and the Mn(II) oxidation site of LiP and MnP, respectively [12,15]. The presence of true LiP or VP enzymes in each fungus showing here AzB or Rb5 bleaching activities, respectively, should be verified when the genomes of these fungi will be available.

Discovery of new potential producers of LiP was noteworthy and one of the main results of this study. In an earlier screening of 68 fungi where laccase and lignin-modifying peroxidase activities were assayed from culture liquids of shake flask cultures and glucose as carbon source, LiP activities were not found, only MnP [45], most probably due to unnatural culture media and conditions. In our study efficiently spruce lignin degrading fungi were grown both in liquid media and in solid lignocellulose media, but we found only seven fungi that showed higher LiP activity than *P.*

chrysosporium ME446, which is a well-known producer of LiP [1]. The most active strains were *Phlebia radiata* 79, *Trametes ochracea* T178, *Daedaleopsis septentrionalis* T20, *Cylindrobasidium evolvens* 58 (syn. *C. laeve*), and *Trametes velutina* K169. Among these the production of LiP has earlier been described in detail only in *P. radiata* 79 [46,47], and it produces at least three LiPs [48]. *D. septentrionalis* T20 and *C. evolvens* 58 have not been reported before as LiP-producing fungi. In contrast, many *Trametes* spp. are known to produce LiP like activities [49,50].

In general, the investigated fungi showed high peroxidase activities when grown in soy medium. Only in the case of LiP activity relatively high enzyme activities were obtained in mineral (M) medium (low-N, "ligninolytic medium"), but in other cases this medium did not support production of laccase or peroxidase activities. Mineral medium (M) is known to support the mineralization of lignin ($^{14}CO_2$ production from ^{14}C-labelled lignin preparations) [1,23] and originally LiP was discovered in *P. chrysosporium* in a very similar medium [1]. The medium is nutrient nitrogen limited with only 2 mM N, which does not allow a high production of any enzyme proteins, but it clearly elicited LiP production. Solid state cultivation on lignocellulose is often suitable for the production of LiP and MnP [42], but it was not superior in our study. However, the enzyme activities in the extracts are difficult to directly compare with the liquid culture supernatants because the mycelial growth is different in solid medium but also because the activity was calculated on the basis of liquid extracted, here 4 g oat husk medium extracted with 16 ml buffer [35], not on the culture filtrates of liquid cultures, which contain less but more easily digestible carbon sources. The use of more nutrient rich media in screening allowed the expression of lignin-modifying peroxidases in many fungi, but apparently the production of LiP was promoted by nutrient poor medium.

Rather few fungi showed the ability to bleach AzB indicating that LiP like activity is rare in nature. This result is consistent with the recent analysis of genome data of 31 fungi by Floudas *et al.* [12] and the data by Morgenstern *et al.* [51], who analyzed more than 150 agaricomycetes and conclude that LiPs evidently arose only once in the Polyporales, whereas MnPs and VPs are more widespread and may have multiple origin. We can summarize that LiP activity was rather rare, fungi produce this activity under nutrient nitrogen limited conditions, and it was only detected when the assay was performed at very acidic condition at pH 3. LiP producing fungi mostly belonged to the order Polyporales and also some to Agaricales, and they were all wood-degrading fungi whereas none of the litter-decomposing fungi produced LiP. Table **1** shows which fungi were selective, i.e. degraded relatively more lignin than cellulose in spruce wood block test [21]. Among the 12 selective fungi all except *P. chrysosporium* ME446, lacked the production of LiP. Also *T. versicolor* showed a high VP-Ind activity when cultivated in soy medium. Another *T. versicolor* strain produces LIPC [49,52], which is presently considered as VP. VP was originally described in a *Pleurotus* sp., and our results indicate that also *P. pulmonarius* K42 is able to produce VP. Rb5 bleaching activity was found in eight other fungi, including *O. leporina* PO119, *P. cinnabarinus*

115 and *C. unicolor* PM170798. Of these fungi, *P. cinnabarinus* has long been considered as a fungus which does not produce LiP or MnP [24]. It is now known that the genome of *P. cinnabarinus* (strain BRFM137) has LiP, VP and MnP encoding genes, but the fungus does not easily express these peroxidases [32]. It constitutively produces laccase, but class II peroxidases are not found in secretomes on hardwood (birch), and only one LiP and one MnP are expressed in different solid state cultures and an atypical VP when maltose is used as carbon source [32]. Apparently soy medium triggered the expression of VP activity in *P. cinnabarinus* in our study. The production of VP was equally common in all major orders studied, and approximately two thirds of the studied species in the orders Agaricales, Polyporales and Hymenomycetales produced VP.

This is the first larger report on the production of neutral MnPs in white-rot fungi, where neutral MnP signifies enzyme(s) that has relatively high activity at pH 7 but it is not necessarily the optimum pH of this activity. The most promising fungi were *D. septentrionalis* T20, *C. borealis* T261, and *Hyphodontia* sp. T165. In oat husk medium *P. velutina* 244i, *P. pulmonarius* K42 and *T. ochracea* T178 were the most efficient producers of MnP activity at neutral pH, while on peptone medium *C. borealis* T261 was efficient. The production of MnPs active at neutral pH thus occurred in a rather large variety of taxonomically different fungi. There are specific applications where laccases and peroxidases active at neutral pH or even at alkaline pH would be desirable, for example in bleaching of paper pulp or in supplement of detergents. As in the case of traditional laundry enzymes, such as proteases, the most promising sources have been bacteria, most often *Bacillus* spp., and peroxidases by these bacteria are reported [53,54]. The ascomycete *Aspergillus terreus* was reported to produce an alkaline peroxidase active at very high pH [55]. Cherry *et al.* [56] developed a *Coprinus cinereus* heme peroxidase mutant suitable for laundry detergent (highly alkaline pH 8.5-10 and oxidative conditions) using directed evolution. Structural characterization of these and the depicted novel pH-tolerant MnPs could further enlighten the structural determinants and critical amino acid residues involved in the protein stability.

Production of Laccases

Laccase is a very common enzyme in wood and litter degrading basidiomycetous fungi [18], but in our assay conditions the ratio of laccase positive samples compared to all samples (with four different media, three sampling times and three assay pHs) studied was rather low: 37% of screening variants were laccase-positive.

Especially in soy medium but also in oat husks fungi rather commonly produced neutral laccase activities (high activity at pH 7), which may indicate that these laccases were active across a rather large pH area, or that these fungi produced different laccase isoenzymes. No laccase showed their pH optimum at pH 7, indicating that white-rot and litter-decomposing basidiomycetes prefer rather acidic conditions. One species, *Pleurotus pulmonarius* K42, possessed a stable laccase that retained activity at pH 7 with 88% of the maximal activity, which occurred at pH 5. Also *Pleurotus ostreatus* DSM11191 and *Physisporinus vitreus* H

retained their laccase activity well at pH 7: 58-57% of the maximal activity (samples of both fungi were obtained from oat husk media). *Kuehneromyces mutabilis* K22, *Cerrena unicolor* PM170798, *Hygrophoropsis aurantiaca* K192, *Trametes pubescens* T65iB, and *Agrocybe praecox* TM70.84 were also noteworthy as producers of laccases with activity at neutral pH. However, these types of laccases seem not to be common compared to the large variety of fungal laccases, *e.g.* ascomycetes laccases such as *Myceliophthora thermophila* laccases, which are much studied and also commercialized. Generally laccases of white-rot fungi have a high redox potential [18], ranging from 730 to 790 mV [57], which allows them to oxidize a much wider range of substrates than low- and medium-redox potential laccases. This property makes them valuable in many applications such as paper pulp bleaching, and therefore basidiomycete laccases that are catalytically active at neutral pH may have advantages in these applications. Some fungi apparently showed high activities in both neutral and very acidic pH. It remains to be studied if they represent same or different isoenzymes.

Efficiency of Automatic Screening

The use of four different media two of which (soy and peptone) were unusual for the search and expression of lignin-modifying oxidoreductases further improved the probability for finding new activities. Especially soy medium appeared to be very efficient in supporting the expression of laccases and lignin-modifying peroxidases by basidiomycetes. The compound(s) in soy flour which elicit the production of enzymes could not be revealed when the fungal peroxygenases were studied [58,59]. Our screening of fungi resulted in several promising candidates for the production of LiP, and novel fungi possessing VP activities were also found. Our results confirmed that MnP is the most common lignin-modifying peroxidase, since only a few fungi did not express this enzyme in any conditions. We could also identify potential fungi producing neutral peroxidases having good activity at pH 7. Laccase was expressed by most fungi studied, and apparently they were high redox potential laccases with acidic pH optima, which is typical for white-rot basidiomycetous fungi. Interestingly, few fungi expressed laccases that were highly active at neutral conditions (pH 7).

The available sequence data of fungal whole genomes is rapidly increasing and many comparative investigations have recently been published [12,25,26,60]. It is now possible to see what kind and how many class II peroxidase and laccase gene models are present in each sequenced fungal genome. However, we cannot yet reliably predict the characteristics of enzymes such as the pH ranges where the enzymes are active. The heterologous production of active class II peroxidases, LiPs, VPs and MnPs, which are heme proteins, is often difficult and has resulted in low yields and only the expression of apoprotein without heme group in both bacterial (e.g. *Escherichia coli*) [61] and eukaryotic (e.g. *Aspergillus* spp.) [62] expression systems. This makes the studies on the expression of lignin-modifying heme peroxidases very tedious and fungal genes cannot be easily expressed in a large extent. Traditional screening of enzyme activities is therefore important, but requires improvements in assay throughput. Altogether, we obtained oxidoreductase

profiles for 53 fungi, and monitored 180 variables per each fungus. Automated screening allowed us to screen many activities and assay conditions from relatively small supernatant or extract volumes. Without pipetting robot the number of different variants had been much lower. When adopting our miniaturized assays, the methods were at first validated using commercial enzymes, and thus, the results can be considered robust and reliable. Robot assisted screening using miniaturized activity assays proved to be a very fruitful approach in the present work.

CONCLUSIONS

MnPs and laccases were most commonly produced in our screening comprising 53 wood-rotting white-rot and litter-decomposing basidiomycetes. The production of LiP and VP, both Mn-dependent and Mn-independent, appeared to be rather rare among the species, and could be detected in 27% of the samples studied. LiP was detected only in certain cultivation media depending on the fungus, whereas MnPs and laccases were readily detected on several media. In this screening we found laccases and MnPs which were stable and active at pH 7. It is noteworthy that LiP and VP activities were detected only when measured at acidic conditions (pH 3). With the aid of automatization and miniaturization it was possible to screen a large number of variants from a small amount of liquid samples. The results were in good agreement with available whole genome studies.

LIST OF ABBREVIATIONS

AzB	=	Azure B
EDTA	=	Ethylenediaminetetraacetic Acid
FBCC	=	Fungal Biotechnology Culture Collection
LiP	=	Lignin Peroxidase
LME	=	Lignin-Modifying Enzyme
MnP	=	Manganese Peroxidase
Rb5	=	Reactive Black 5
VP	=	Versatile Peroxidase
VP-Ind	=	Manganese Independent Versatile Peroxidase
VP-Mn	=	Manganese Dependent Versatile Peroxidase

CONFLICT OF INTEREST

The authors confirm that there are no conflict of interest.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's web site along with the published article.

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SUPPLEMENTARY MATERIAL

Improved Efficiency in Screening for Lignin-Modifying Peroxidases and Laccases of Basidiomycetes

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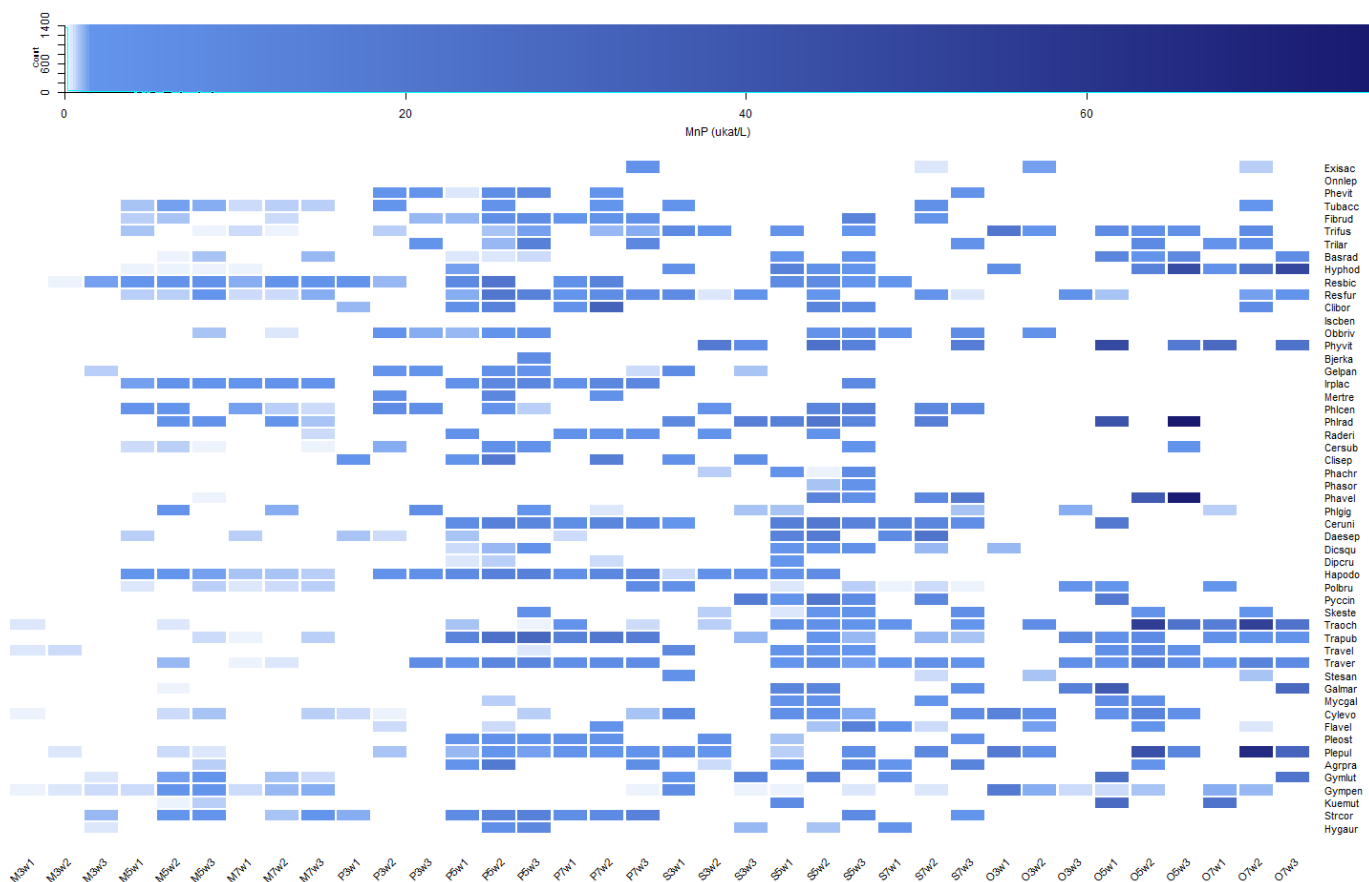
Fig. (S1). Heatmap of MnP activities (μkat/L) detected from fungal culture supernatants from mineral (M), peptone (P) or soy (S) liquid media or extract of cultivation on oat husks (O). MnP was assayed at buffer pH 3 (3), pH 5 (5) or pH 7 (7). Culture duration indicated in weeks (w1, w2, w3). The ordering of the corresponding rows and hence the abbreviations of fungal species is identical to Table **1**. Figure was plotted using R [1].

Fig. (S2). Heatmap of laccase activities (μkat/L) detected from fungal culture supernatants from mineral (M), peptone (P) or soy (S) liquid media or extract of cultivation on oat husks (O). Laccase was assayed at buffer pH 3 (3), pH 5 (5) or pH 7 (7). Culture duration indicated in weeks (w1, w2, w3). The figure was drawn by R package gplots. Ordering of the corresponding rows and hence the abbreviations of fungal species is identical to table 1. Figure was plotted using R [1].

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