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AGRO-FORESTRY RESIDUES VALORIZATION BY LIGNINOSOME OF *GRIFOLA FRONDOSA*

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ABSTRACT

Grifola frondosa HAI 1232 was tested for ligninolytic enzyme activities and for lignin, cellulose and hemicellulose degradation during cultivation on eight common agro-forestry residues in Serbia. Wheat straw was favorable lignocellulosic for the production of Mn-dependent and Mn-independent peroxidases (2513.89 and 354.17 U L^{-1}, respectively), while selected residues inhibited the synthesis of laccases. The highest lignin removal was observed during fermentation of blackberry sawdust (36.75%), while the highest selectivity index was recorded on oak sawdust (4.34). The dry matter loss varied between 8.17% in corn stalks and 14.16% in apple sawdust. According to the presented results, it can be concluded that *G. frondosa* HAI 1232 could be an important participant in various biotechnological processes due to its high capacity to selectively degrade different agro-forestry residues.

KEYWORDS: Agro-forestry residues, delignification, *Grifola frondosa*, ligninolytic enzymes.

INTRODUCTION

In the future bio-based society, global challenges such as climate changes, ecosystem degradation, associated with industrialization and growing global population, force us to find effective biological solutions. Fungi are relatively understudied, but according to Hyde et al. (2019) they are an essential and biotechnologically useful group of organisms with a remarkable potential for industrial exploitation. It is known that many modern industries are based on fungi and their metabolites and it is believed that these organisms are the future cell factories for the production of food, pharmaceuticals, enzymes and bio-control agents (Goyal et al. 2016, Lange et al. 2020, Meyer et al. 2020).

Reducing dependence on non-renewable, unsustainable resources is one of the keys for stable economic growth and sustained environmental quality (Antov et al. 2020). Industrial and

agricultural wastes, rich in lignocellulose, are produced in large tonnages and have special importance because of their renewable nature (Asgher et al. 2013). However, majority of these residues are either improperly deposited or disposed of by burning, which is not restricted to developing countries alone. Ghaffar et al. (2015) also pointed out that enormous amounts of cuttings from fruits remain unexploited and present serious ballast of the environment. Thus, based on the data from Food and Agriculture Organization, reported by Krausmann et al. (2008), 6.6 G ton of dry biomass is unused per year of which a 2.5 G ton are burned residues. Although collecting of that biomass and its conversion to valuable products will require much efforts and energy, Bos and Broeze (2020) indicate that these biotransformations may significantly contribute to sustainability goals. A good example of such a contribution is the development of the new biorefinery which was started with the conversion of highly abundant wheat straw and corn stover to biofuel, as one of the most widely used alternative energy sources (Ghaffar et al. 2015, Lange et al. 2020).

Over the last 5-10 years, fungi have taken the center role in the conversion of lignocellulosic residues into different high-value products. Physical, chemical and physico-chemical pretreatments of the residues employ methods which are cost/energy intensive, while biological one including the white-rot fungi, is mild and environment friendly. It is well known that fungal enzymes are always preferred over plant and animal ones due to wide isoforms diversity and extraordinary catalytic properties. Furthermore, their low cost, faster production and higher yield are some more of the advantages (Goyal et al. 2016). Through the action of a strong ligninolytic enzyme system, containing lignin and Mn-oxidizing peroxidases, laccases and some auxiliary enzymes, white-rot fungi can degrade lignin, the most recalcitrant polymer in biomass (Saritha and Arora 2012, Ćilerdžić et al. 2017). Because of strong bond to hollocellulose and role of protective barrier, lignin increases resistance of cell wall and complicates the accessibility of cellulolytic enzymes to cellulose which requires decomposition of the lignin network prior hydrolysis (Esteghlalian et al. 2001, Hideno et al. 2007, Dashtban et al. 2009, Saritha and Arora 2012). Controlling the number factors which affect the activities of the enzymes and lignin degradation extent, such as fungal species/strain, lignocellulose type and cultivation conditions leads to the most effective pretreatment process (Isroi et al. 2011, Ćilerdžić et al. 2017). Fungal delignification integrated with solid-state production of low-cost cellulase would result in a cost-efficient conversion of lignocellulose materials (Saritha and Arora 2012). Fungal role in biomass conversion is of great significance because they can “unlock” the full potential of the lignocellulosic biomass which expands the range of the fungal applications (Hyde et al. 2019).

Due to the unique nutritional and medicinal properties of *G. frondosa*, which have been a topic of numerous researches, this white-rot mushroom has gained popularity as a healthy food. However, not much data is available on its ligninolytic enzymes potential. Considering its availability and cultivation on a commercial scale, further research is needed to broaden the knowledge of this significant species. Therefore, the present study aimed to determine Mn-oxidizing peroxidases and laccase activity profiles in *G. frondosa* cultivated on common agro-forestry residues and its potential for delignification.

MATERIALS AND METHODS

Organism and cultivation conditions

The culture of *Grifola frondosa* HAI 1232, obtained from the Institute of Evolution, University of Haifa, Israel (HAI) and deposited in the collection of the Faculty of Biology, University of Belgrade was used in this study. The inoculum was prepared in 250 mL flasks containing 100.0 mL of synthetic glucose/ammonium nitrate/yeast extract medium inoculated with 7-days old "mother" culture. The incubation was conducted at room temperature on a rotary shaker for 7 days. Sterile distilled water (dH_2O) was used to wash the obtained biomass and its homogenization was performed in a laboratory blender (Waring, USA). Solid-state fermentation of 8 agro-forestry residues (apple-, blackberry-, grapevine-, oak-, plum-, and raspberry sawdust, corn stalks and wheat straw) was carried out in 250 mL Erlenmeyer flasks, containing 6.0 g of the residue soaked with 30.0 mL of the modified synthetic medium (without glucose) and inoculated with 9.0 mL of the homogenate. The cultivation was conducted at 25°C in the dark for 21 days. All the experiments were done in triplicates and results were expressed as mean ± standard error.

Assays of enzyme activity and total protein production

According to the method of Stajić et al. (2017), the produced ligninolytic enzymes were extracted by sample stirring with 50.0 mL of dH_2O at 4°C for 10 min. The obtained extracts were centrifugated (4°C, 3000 rpm, 15 min) and resulting supernatants were used for measurement of MnP-oxidizing peroxidases [Mn-dependent peroxidase (MnP, EC 1.11.1.13) and Mn-independent peroxidase (MnIP, EC 1.11.1.16)] and laccase (EC 1.10.3.2)], as well as total protein content by a spectrophotometer [CECIL CE2501 (BioQuest), UK]. Mn-oxidizing peroxidases activities were determined by the rate of oxidation of Phenol Red at 610 nm. The reaction mixture (V_{tot} = 1.0 mL) contained succinate buffer, sample, 2mM H_2O_2, and phenol red, with or without addition of $MnSO_4$ (for MnP and MnIP, respectively). Laccase activity was assayed in the mixture composed of phosphate buffer, ABTS (2,2'–azinobis (3–ethylbenzothiazoline–6–sulfonicacid), and sample, at 436 nm. Enzymatic activity was expressed in U L^{-1}, and the activity of 1U presents the amount of enzyme that transforms 1.0 μmol of substrate per min.

The total protein content (mg mL^{-1}) was determined by the method of Silva et al. (2005), i.e. by measurement of Bradford's reagent color change at λ = 595 nm induced by sample. The bovine serum albumin was used as the standard and further for calculation of the specific enzyme activity (U mg^{-1}).

Determination of lignin, cellulose and hemicellulose contents

The loss of substrate dry matter (%) was determined by Eq. 1:

$$(Mi - Mf)/Mi \times 100 \quad (1)$$

where: M*i* - the initial lignocellulosic mass, M*f* - the mass after fermentation.

The determination of hemicellulose, cellulose and lignin contents was carried out using the modified methods of Kirk and Obst (1988) and Van Soest et al. (1991). Dried ground sample (1.0 g) was treated with neutral detergent/Na_2SO_3 mixture aiming to remove soluble sugars, proteins, lipids, and vitamins and the obtained biomass presented neutral detergent fibers (NDF). Hemicellulose was removed with a solution of acidic detergent and the obtained mass was defined as acidic fibers (ADF). The hemicellulose amount was determined as difference between NDF and ADF. Lignin content (LC) expressed as the percentage of quantity presented in the initial sample was defined after ADF incubation with 72% H_2SO_4 at 30°C and hydrolysis at 120°C. Cellulose content represented the difference between ADF and LC. Selectivity index, *i.e*. lignin/cellulose removal ratio was used as an indicator of lignin degradation selectivity.

Statistical analyses

All the experiments were done in three replicates and the results were expressed as mean ± standard error. Assaying of any significant differences among means was performed by One-way analysis of variance (ANOVA) and Tukey`s test, using STATISTICA, version 6.0 (StatSoft, Inc., Tulsa, USA). Statistical significance was declared at $p < 0.05$.

RESULTS AND DISCUSSION

During 21 days of solid-state cultivation, *G. frondosa* HAI 1232 synthesized assayed enzymes which activity depended on the carbon source i.e., agro-forestry residues (Fig. 1). Thus, wheat straw was favorable lignocellulosic for the production of MnP by studied species as a very high activity was detected (2513.89 U L^{-1}). The moderate levels of the enzyme activity were obtained after oak sawdust and corn stalks fermentation (87.12 U L^{-1} and 60.61 U L^{-1}, respectively), while it was remarkable lower after fermentation of plum, raspberry and grapevine sawdust, ranging from 29.04 U L^{-1} to 47.98 U L^{-1} and even absent on apple- and blackberry sawdust (Fig. 1).

Regarding MnIP activity, it showed greater variation in dependence on tested residues than MnP. Although the maximum activity was also detected on wheat straw, its peak was many-fold lower (354.17 U L^{-1}) than in the case of MnP (Fig.1). Blackberry sawdust also induced MnIP (142.05 U L^{-1}), while two-fold and lower values of enzyme activity were observed on other substrates. The minimal MnIP activity was noted on apple sawdust (9.47 U L^{-1}), while grapevine sawdust inhibited the synthesis of this enzyme (Fig. 1).

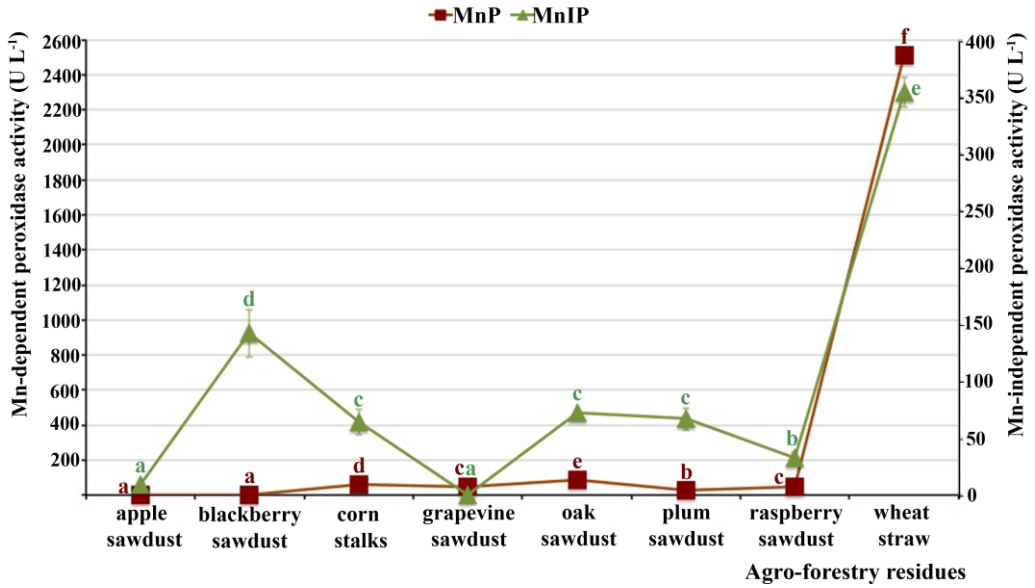

Fig. 1: Mn-dependent and Mn-independent peroxidase activity of Grifola frondosa HAI 1232 during agro-forestry residues fermentation. Values superscripted with the same letter in each values group (for each enzyme) are not significantly different ($p < 0.05$).

Contrary to Mn-oxidizing peroxidase activities, selected residues completely inhibited the laccase activity, which could be explained by strong influence of fermentation period on the activity. Total protein concentration was ranged from 0.63 mg mL^{-1} after raspberry fermentation to 2.99 mg mL^{-1} noted on corn stalks, which consequently reflected on specific enzyme activities. Thus, the maximal MnP specific activity was detected after wheat straw fermentation (2.07 U mg^{-1}), while the activities for other tested enzymes were less than 1.0 U mg^{-1}.

The lignocellulose degradation ability of *G. frondosa* HAI 1232 was also evaluated (Tab. 1). The results clearly showed that this species was good and selective degrader of most tested agro-forestry residues. Thus, the highest total dry matter loss was recorded in raspberry sawdust, even 14.16%. The reduction of dry matter was also significant in apple- and blackberry sawdust (13.83% and 13.17%, respectively), while the lowest values were obtained for corn stalks (8.17%) and oak sawdust (8.30%). Considering the laccase absence during wheat straw fermentation, Mn-oxidizing peroxidases could be responsible for lignin removal (30.82%), which degradation rate was in accordance with the activity level (Fig. 1, Tab. 1). However, this correlation was not observed for other substrates. A slightly higher delignification extent was noted during blackberry sawdust and corn stalks fermentation (36.75% and 32.26%, respectively). *G. frondosa* weakly depolymerized cellulose in these three residues which reflected on high selectivity indices descending from 3.39 in corn stalks, through 2.15 in blackberry sawdust to 1.97 in wheat straw. However, the highest selectivity index (4.34) was obtained after oak sawdust fermentation, but lignin depolymerization was not highly effective (23.85%). The lowest selectivity indices were noted during cultivation of grapevine- and plum sawdust (1.02 and 1.16, respectively) where the capacities of lignin and cellulose degradation were similar, while hemicellulose mineralization was remarkable, 46.29% in plum sawdust and 35.83% in grapevine sawdust (Tab. 1).

Tab. 1: The level of agro-forestry residues depolymerization (%) by Grifola frondosa (values superscripted with different letters in the same column are significantly different (p < 0.05).

Agro-forestry residues	Samples	Sample weight (g)	Polymers composition of samples (mg)			Dry matter loss (%)	Extent of polymers degradation (%)			Sel. index
			Lignin	Cellulose	Hemicel.		Lignin	Cellulose	Hemicel.	
Apple sawdust	Control*	6.00	1158.00	2808.00	1176.00	/	/	/	/	/
	HAI 1232	5.17	852.72	2253.25	821.71	13.83^{c}	26.36^{c}	19.76^{e}	30.13^{d}	1.33^{a}
Blackberry sawdust	Control*	6.00	1218.00	2712.00	1038.00	/	/	/	/	/
	HAI 1232	5.21	770.40	2248.56	811.98	13.17^{c}	36.75^{e}	17.09^{d}	21.77^{b}	2.15^{b}
Corn stalks	Control*	6.00	594.00	2796.00	1860.00	/	/	/	/	/
	HAI 1232	5.51	402.38	2530.11	1504.78	8.17^{a}	32.26^{d}	9.51^{b}	19.12^{b}	3.39^{c}
Grapevine sawdust	Control*	6.00	1421.41	2652.00	887.08	/	/	/	/	/
	HAI 1232	5.27	1201.79	2250.72	569.27	12.16^{b}	15.45^{a}	15.13^{c}	35.83^{e}	1.02^{a}
Oak sawdust	Control*	6.00	1530.00	2808.00	1159.00	/	/	/	/	/
	HAI 1232	5.50	1165.15	2654.57	802.42	8.30^{a}	23.85^{b}	5.49^{a}	30.82^{d}	4.34^{d}
Plum sawdust	Control*	6.00	1837.49	2544.00	1368.00	/	/	/	/	/
	HAI 1232	5.29	1464.22	2098.54	734.45	11.83^{b}	20.31^{b}	17.51^{d}	46.29^{f}	1.16^{a}
Raspberry sawdust	Control*	6.00	1200.00	2160.00	1308.00	/	/	/	/	/
	HAI 1232	5.15	875.16	1930.50	988.42	14.16^{c}	27.07^{c}	10.63^{b}	24.43^{c}	2.54^{b}
Wheat straw	Control*	6.00	666.00	2418.00	1692.00	/	/	/	/	/
	HAI 1232	5.30	460.75	2038.96	1488.18	11.66^{b}	30.82^{d}	15.68^{c}	12.05^{a}	1.97^{b}

Comparing to some previous reports, *G. frondosa* strain profiled in this study has shown a better ability to produce highly active Mn-oxidizing peroxidases. Thus, Montoya et al. (2012) reported very low MnP activity (4 U g^{-1}) after 23 days of solid-state fermentation of oak sawdust/corn bran medium by *G. frondosa* PSUMCC 922, while slightly higher activity (22.6 U L^{-1}) was observed by Mikiashvili et al. (2011) after 35 days of oak sawdust solid-state fermentation by strain MBFBL 684. However, contrary to our strain that did not produce laccases, the strains studied by other authors secreted laccase during fermentation of various lignocellulosics. Thus, strain studied by Montoya et al. (2012) produced low active laccase (12 U g^{-1}) after 23 days of oak sawdust/corn bran medium fermentation. Contrary to Xing et al. (2006) who reported activity of only 70 U L^{-1} after even 52 days of submerged cultivation in glucose/ammonium nitrate/yeast extract/asparagine monohydrate medium, strain MBFBL 596 studied by Mikiashvili et al. (2011) secreted laccase of even 703.3 U L^{-1} after only 15 days of oak sawdust solid-state fermentation. This could be explained either by the fact that laccase activity decreases with cultivation period which was already shown in previous reports (Ćilerdžić et al. 2014, Stajić et al. 2017) or by the influence of medium composition.

Despite the lack of laccase activity, *G. frondosa* HAI 1232 was more effective delignifier than other previously tested species/strains of this genus as well as other white-rot fungi. Thus, Mikiashvili et al. (2011) reported much lower lignin mineralization (< 10%) during oak sawdust

fermentation by studied *G. frondosa* strains. Although our strain was less effective in lignin removal from oak sawdust than strain tested by Montoya et al. (2012), which degraded 67% of lignin, our strain showed higher selectivity. In comparison with white rot species studied by Zhao et al. (2020), strain HAI 1232 showed higher delignification capacity and selectivity during corn stalks depolymerization. However, *Pleurotus* species studied by Ćilerdžić et al. (2017) were much better and more selective delignifiers of wheat straw than *G. frondosa* HAI 1232.

CONCLUSIONS

Although fungi are essential for matter cycling due to their degradative abilities and have so many proven uses in different biotechnological areas, their great potential needs further exploration. Screening fungi for the production of ligninolytic enzymes and consequently delignification of various agro-industrial residues has often been reported, but to date, the ligninolytic potential of *G. frondosa* has only scarcely been researched. Therefore, the presented results have clearly shown that *G. frondosa* HAI 1232 is a potent degrader of frequent agro-forestry residues. Demonstrated high capacity of studied species to selectively degrade lignocellulose also indicated its ability to potentially participate in various biotechnological processes. Since the use of fungi and fungal products is considered very important for a more sustainable future, these promising results present one more contribution to the reaching of this goal.

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