CAPACITY OF *AURICULARIA AURICULA-JUDAE* TO DEGRADE AGRO-FORESTRY RESIDUES

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Agro-forestry residues are a widespread lignocellulosic biomass, which could be used as raw material in many industries. The essential precondition for their usage is delignification, a process where white-rot fungi have a crucial role due to their powerful ligninolytic enzyme system. The objectives of this study were to investigate the insufficiently studied ligninolytic potential of *Auricularia auricula-judae* by determining its Mn-oxidizing peroxidase and laccase activities, as well as its capacity for lignin, hemicellulose and cellulose degradation during fermentation of common agro-forestry residues. The highest Mn-dependent and Mn-independent peroxidase activities were detected after plum sawdust and wheat straw solid-state fermentation (121.21 U L^{-1} and 136.36 U L^{-1}, respectively), while only blackberry sawdust induced laccase production (107.37 U L^{-1}). A significant reduction in the lignin content (46.4%) was achieved after wheat straw fermentation, while the highest selectivity index (12.71) was observed for oak sawdust. These data show that *A. auricula-judae* has a great potential to delignify plant residues and, consequently, can be used in various biotechnological processes.

Keywords: *Auricularia auricula-judae*, plant residues, delignification, laccases, Mn-oxidizing peroxidases

INTRODUCTION

The continuous growth of the human population and intensive industrial development lead to an increased need for food and energy, as well as to a reduction of natural resources and numerous harmful effects on the environment. Therefore, examining the potential of renewable lignocellulosic biomass to be used as an economically and ecologically justified alternative energy source, and defining the most effective ways of its processing represent topics of great research interest in the 21st century.[1] Agricultural and forestry residues are some of the most abundant and the cheapest energy resources, whose usage can significantly decrease the exploitation of fossil fuels and the negative impact on the environment.[2,3] However, despite the high potential of these residues, not only for energy production, but also for food, feed, paper and various chemicals production, they usually remain unutilized and present a serious burden for nature.[4]

The transformation of lignocellulose, composed of lignin, cellulose, hemicellulose and small amounts of other components, is still a great challenge because of its complex structure and, consequently, high stability and resistance to degradation. Namely, lignin is one of the most resistent natural compounds and delignification, *i.e.* pretreatment, represents a crucial step in efficient conversion of lignocellulose into valuable products.[5] Although various chemical, physical and physico-chemical methods for delignification are available, the release of numerous hazardous by-products, significant energy consumption and, consequently, high cost are the main disadvantages of these pretreatments. Therefore, biological pretreatment, characterized by very low energy consumption, minimal waste production and lack of any side effect on the environment, represents an alternative method.[6] However, this method also has some disadvantages, which limit its application, such as the long period of time and large space required for its realization.[7] That is why, nowadays, special emphasis is put on the creation of efficient biological delignification systems, *i.e.* finding the most efficient organism and defining of the optimal cultivation conditions for good and highly selective pretreatment of certain lignocellulosic residues.

Although the highest efficiency of biological pretreatment has been achieved by white-rot fungi, owing to their well-developed ligninolytic enzymatic system, it is known that the enzyme production and activity can be different not only among species, but also among strains of the same species, *i.e.* they are regulated genetically and by abiotical factors (carbon and nitrogen sources, temperature, pH, humidity, level of aeration *etc.*).[3,8] The benefit of using white-rot fungi also lies in their ability to

selectively decompose lignocellulose, which leads to remarkable lignin loss, leaving cellulose and hemicellulose, which can be used as sources for production of biofuel and numerous chemicals.[9-11]

Auricularia auricula-judae is a well-known edible and medicinal mushroom, containing numerous bioactive compounds.[12] However, though it is known as a white-rot species, its ligninolytic potential is still insufficiently studied. Therefore, in this work, we intended to contribute to the understanding of the *A. auricula-judae* ligninolytic system and its ability to degrade raw plant materials. Based on this, the objective of the study was defined: to determine the activity of Mn-oxidizing peroxidase and laccase, as well as the level of lignin, cellulose and hemicellulose degradation by *A. auricula-judae* during solid-state fermentation of eight abundantly available agro-forestry residues.

EXPERIMENTAL

Organism and cultivation conditions

The strain of *A. auricula-judae* HAI 330 was received from the culture collection of the Institute of Evolution, University of Haifa, Israel (HAI), and maintained on malt agar medium at 4 °C, in the culture collection of the Institute of Botany, Faculty of Biology, University of Belgrade.

The inoculum was prepared by inoculation of 100.0 mL of a synthetic medium (glucose, 10.0 g L^{-1}; NH_4NO_3, 2.0 g L^{-1}; K_2HPO_4, 1.0 g L^{-1}; $NaH_2PO_4 \cdot H_2O$, 0.4 g L^{-1}; $MgSO_4 \cdot 7H_2O$, 0.5 g L^{-1}; yeast extract, 2.0 g L^{-1}; pH 6.5) with 25 mycelial agar discs (Ø 0.5 cm) of 7-day old culture, and incubation at room temperature (22 ± 2 °C) on a rotary shaker (100 rpm) for 7 days. The obtained biomass was washed three times with sterilized distilled water (dH_2O) and homogenized in a laboratory blender (Waring, USA). The thus-prepared inoculum, in the amount of 9.0 mL, was used for inoculation of substrates composed of 6.0 g of selected plant residues (wheat straw, raspberry, blackberry, plum, apple, oak and grapevine sawdust, as well as corn stalks) and 30.0 mL of the modified synthetic medium (without glucose). Solid-state fermentation of the raw plant materials was carried out in 250 mL flasks, at 25 °C, during 21 days.[13]

Assays of enzymatic activity

According to the methods of Stajić *et al*.,[13] the extraction of produced extracellular enzymes was carried out by stirring the sample with 50.0 mL of dH_2O at 4 °C for 10 min. The thus-obtained extracts were centrifuged (4 °C, 3000 rpm, 15 min) and the resulting supernatants were used for measurement of Mn-oxidizing peroxidase and laccase activities, as well as of total protein content spectrophotometrically [CECIL CE2501 (BioQuest), UK]. The activity of Mn-oxidizing peroxidases [Mn-dependent peroxidase (MnP, EC 1.11.1.13) and Mn-independent peroxidase (MnIP, EC 1.11.1.16)] was estimated by monitoring the A_{610} change related to the rate of phenol red oxidation (ε_{610} = 22 000 M^{-1} cm^{-1}), while 2,2′-azino-bis-[3-ethylthiazoline-6-sulfonate] (ABTS) (ε_{436} = 29 300 M^{-1} cm^{-1}) was used as substrate for defining the activity of laccase (Lac, EC 1.10.3.2). The peroxidase activities were determined after stopping the reaction in the reaction mixture [succinic buffer, sample, 2.0 mM H_2O_2 and phenol red, with or without 2.0 mM $MnSO_4$ (for MnP and MnIP, respectively); V_{tot} = 1.0 mL] by addition of 2 M NaOH. The reaction mixture for defining laccase activity was composed of phosphate buffer, ABTS and the sample, and the reaction was carried out at 35 °C. Enzymatic activity of 1 U is defined as the amount of enzyme that transforms 1 μM of substrate per min.

The content of total proteins (mg mL^{-1}) was determined by monitoring the evolution of A_{595} in the reaction mixture composed of Coomassie brilliant blue G-250 and sample, and by using the standard curve obtained from solutions of bovine serum albumin (0.00, 0.01, 0.02, 0.03, 0.05 0.1, 0.2, 0.3, 0.5, 0.8 or 1.0 mg mL^{-1}) and a Bradford reagent.[4]

Determining hemicellulose, cellulose and lignin contents

The total dry matter loss (%) was calculated according to the equation:

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

where M*i* – initial mass of the lignocellulosic substrate, M*f* – final mass of the lignocellulosic substrate after the fermentation period.

The determination of hemicellulose, cellulose and lignin content was performed using the modified methods of Kirk and Obst[14] and Van Soest *et al*.[15] According to Van Soest *et al*.,[15] soluble sugars, proteins, lipids and vitamins were removed by treatment of the dried ground sample (1.0 g) with a neutral detergent/Na_2SO_3 solution under refluxing conditions, and the obtained biomass presented neutral detergent fibers (NDF). Hemicellulose was removed with a solution of acidic detergent under refluxing conditions and the content of acidic detergent fibers (ADF) was measured. The hemicellulose content represented as NDF – ADF. Lignin content (LC) was defined by the Klason method[14] by incubation of the sample in 72% H_2SO_4 at 30 °C and its hydrolysis at 120 °C, and expressed as the percentage of lignin amount in the original sample. Cellulose content was calculated as ADF – LC.

The selectivity index, a key parameter for characterizing the selective capability of lignin degradation, was

defined as the ratio between lignin and cellulose removal.

Statistical analyses

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

RESULTS AND DISCUSSION

The obtained results have clearly shown that *Auricularia auricula-judae* HAI 330 possesses a remarkable ligninolytic potential, based primarily on the production of highly active Mn-oxidizing peroxidases after 21-day long cultivation on all the tested substrates. As opposed to peroxidases, laccase activity was recorded only after fermentation of blackberry sawdust (Figs. 1, 2). However, despite the high potential to produce peroxidases, MnP and MnIP activities depended on the type and chemical composition of the plant residues. Thus, the highest MnP activity (121.21 U L^{-1}) was noted after *A. auricula-judae* cultivation on plum sawdust, slightly lower after grapevine sawdust and corn stalks fermentation (107.32 U L^{-1} and 100.38 U L^{-1}, respectively), while a sudden activity decline was detected on blackberry and oak sawdust (activity was about 10.00 U L^{-1}) (Fig. 1). Wheat straw was the best inducer of MnIP activity, which reached a value of 136.36 U L^{-1}. Blackberry and plum sawdusts were also good substrates for synthesis of highly active isoforms of this enzyme (125.00 U L^{-1} and 102.27 U L^{-1}, respectively), while during *A. auricula-judae* cultivation on raspberry and apple sawdusts, the minimal values of the enzyme activity were noted (30.30 U L^{-1} and 26.52 U L^{-1}, respectively) (Fig. 1). Contrary to Mn-oxidizing peroxidases, no laccase activity was reported after 21 days of solid-state fermentation of 7 out of 8 tested plant residues. Namely, the blackberry sawdust was the only raw plant material that stimulated the synthesis of relatively high active laccase (107.37 U L^{-1}) (Fig. 1).

Although, the highest amount of total produced proteins was noted after wheat straw and corn stalks fermentation (1.71 mg mL^{-1} and 0.65 mg mL^{-1}, respectively), the maximal specific MnP, MnIP and Lac activities (0.154, 0.125 and 0.108 U mg^{-1}, respectively) were detected after 3-week long *A. auricula-judae* cultivation on corn stalks and blackberry sawdust based substrates.

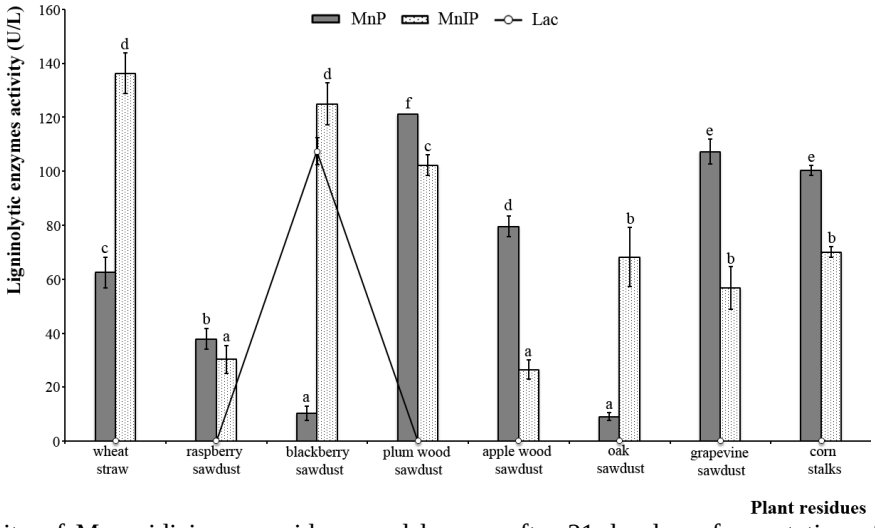

Figure 1: Activity of Mn-oxidizing peroxidases and laccase after 21-day long fermentation of selected plant residues by *Auricularia auricula-judae* HAI 330 (the values with different superscripts differ significantly ($p < 0.05$) according to Tuckey's test

Table 1
Dry mass loss and depolymerization selectivity of studied plant residues after fermentation with *Auricularia auricula-judae*

Plant residues	Total dry matter loss (%)	Selectivity index
Wheat straw	16.80 ± 0.83	3.22
Raspberry sawdust	20.76 ± 0.22	4.20
Blackberry sawdust	17.34 ± 0.84	2.76
Plum wood sawdust	17.73 ± 0.28	0.73
Apple wood sawdust	17.70 ± 0.30	1.48
Oak sawdust	13.42 ± 0.79	12.71
Grapevine sawdust	14.66 ± 0.77	1.93
Corn stalks	15.26 ± 0.41	1.75

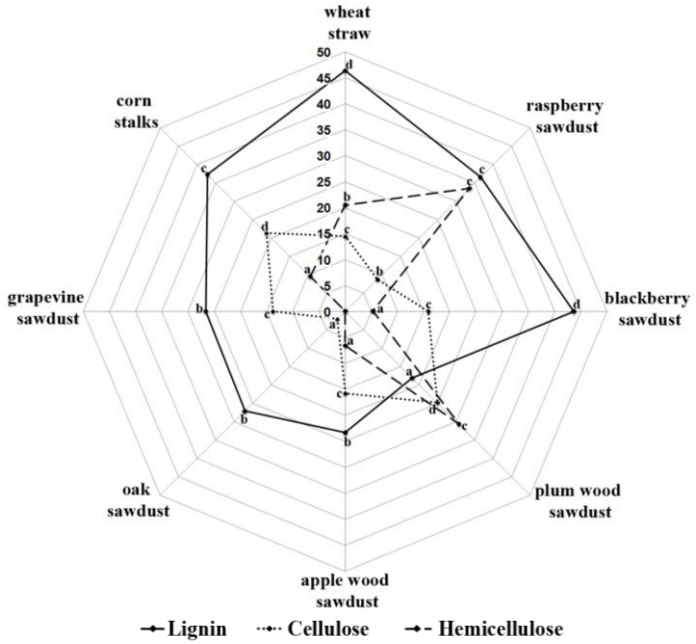

Figure 2: Level of plant residue depolymerization (%) by *Auricularia auricula-judae* HAI 330

It should be emphasized that this study is one of the rare works performed on the ligninolytic enzyme system of this species. Based on scarce literature on this topic, primarily about Mn-oxidizing peroxidases, *A. auricula-judae* is classified in the group of white-rot fungi.[16] Namely, Liers *et al.*[16] reported that highly active forms of MnP and MnIP were synthetized during beech wood fermentation with the peak of activity on day 36 (up to 1750.00 U L^{-1}) and day 24 (up to 335.00 U L^{-1}), respectively. These remarkably higher values than those achieved with the strain HAI 330 can be explained by differences in the substrate type and period of cultivation. On the other hand, despite the low laccase activity of the strain HAI 330, it was more than 5-fold higher than the activity noted by Liers *et al.*[16] However, it should not be concluded that the tested substrates do not induce laccase synthesis, as the cultivation period can also be the reason for the lack of its active form. Generally, it is known that the activity of white-rot species laccases gradually increases at the beginning of cultivation with a peak after 7-10 days, and afterwards it begins to decline sharply.[17,18] Thus, Ma *et al.*[17] noted the maximum laccase activity in *Auricularia polytricha* on day 5 (1686.00 U g^{-1}), which gradually decreased over the cultivation period, and completely stopped on day 15.

Owing to the well-developed ligninolityc enzyme system, the studied strain HAI 330 was not only a good degrader of the tested lignocellulosic residues, but also a highly selective one (Table 1, Fig. 2). The total dry matter loss in the studied plant raw materials ranged between 13.42% in oak sawdust and 20.76% in raspberry sawdust (Table 1). Regardless of the expectations that higher enzyme activities lead to a higher depolymerization degree, the correlation between the enzymatic activity and the

delignification level was not achieved for all the residues. It can be explained by the selected measurement point, when the activity of laccase was almost not detected. The highest level of lignin content reduction (46.4%), and a high selectivity index in fiber depolymerization (3.22) were noted after wheat straw fermentation with *A. auricula-judae* (Table 1, Fig. 2). A slightly lower extent of delignification (43.67%) and a similar selectivity index (only 15.8% of cellulose was depolymerized) were obtained after blackberry sawdust fermentation. In the case of oak sawdust, an extremely high selectivity index (12.71) was noted although the lignin loss was only 27.2%, while the plum sawdust was the most resistant to the enzymes of this strain, which mineralized only 18.1% of the lignin and recorded the lowest degradation selectivity (0.73) (Table 1, Fig. 2).

Althouth there are numerous results on plant residue degradation by various fungal species (*Daedaleopsis* spp., *Ganoderma* spp., *Trametes* spp. *etc*.), data about the ligninolytic potential of *Auricularia* spp. are very scarce. Also, the majority of the studied lignocellulosic residues were researched for the first time, though they are abundantly available in Europe. From our review of the existing literature on the topic, only Ma *et al*.[17] reported on the ability of *A. polytricha* to mineralize the lignin of corn stover. However, a comparison of the results showed that *A. auricula-judae* HAI 330 is a much stronger lignin degrader than *A. polytricha*, which degraded only 16.6% of lignin after 15 days of corn stover fermentation.

CONCLUSION

The presented results have clearly shown that *Auricularia auricula-judae* HAI 330 could be an important participant in the pretreatment of lignocellulosic residues due to its well-developed enzymatic system and high selectivity in lignin degradation. Likewise, this study demonstrated that the type and composition of the residues strongly affect the delignification extent. The obtained data could be applied in many industrial branches, such as paper and biofuel production, but also in food production, namely, in the production of nutritionally and medicinally high-valued fruiting bodies of this species.

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