POTENTIAL OF *TRAMETES HIRSUTA* TO PRODUCE LIGNINOLYTIC ENZYMES DURING DEGRADATION OF AGRICULTURAL RESIDUES

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Trametes hirsuta is an efficient lignin-degrading species due to its ability to produce laccase and Mn-dependent peroxidase. Agricultural residues represent prospective substrates for the bioconversion into fungal biomass and lignocellulolytic enzymes, but also they could be potential environmental pollutants. Evaluation of the potential of *T. hirsuta* to produce ligninolytic enzymes during solid-state fermentation of selected plant raw materials was the goal of the study. The highest level of laccase activity (3827.0 ± 219.0 U/L) was noted in mandarin orange peels medium, while wheat straw and glucose-enriched mandarin orange peels media were the optimum for Mn-dependent peroxidase (1971.5 ± 23.0 U/L) and versatile peroxidase (1173.0 ± 100.0 U/L), respectively. Levels of veratryl alcohol oxidation in the presence of H_2O_2 were similar to those in its absence during fermentation of all selected agricultural residues, and the highest values were obtained in glucose-enriched mandarin orange peels medium. The obtained results demonstrated the potential of *T. hirsuta* to produce ligninolytic enzymes and mineralize various plant wastes to low-molecular weight compounds that could be further used in food, feed, and other industries.

Keywords: Agricultural wastes; Laccase; Mn-oxidizing peroxidases; Trametes hirsuta; Veratryl alcohol oxidation

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INTRODUCTION

Trametes species are known as some of the most efficient lignin-degrading species due to their ability to produce lignin-modifying enzymes. These enzymes enable degradation of lignin and a wide range of compounds with structural similarities to lignin, in addition to the reported resistance of *Trametes* species to toxic or mutagenic chemicals (Peláez et al. 1995; Champagne and Ramsay 2005; Osma et al. 2007). Ligninolytic enzyme systems could also play important role in various processes, such as biopulping, biobleaching, textile dye discolouration, treatment of agricultural residues, and industrial wastewater, etc., and contribute to solving pollution problems (Moldes et al. 2003; Rodriguez Couto et al. 2006; Rosales et al. 2007). Recently, agricultural and wood wastes have been produced in enormous amounts worldwide. Due to the very expensive and inefficient process of their chemical and physical degradation, biological decomposing is more acceptable and feasible, and presents a popular alternative for the treatment of various wastes for both economic and ecological reasons (Croan 2000). Nowadays,

conversion of plant wastes to low-molecular weight compounds by fungal lignocellulolytic enzymes is a basis for production of high nutrition valued food (mushroom fruiting bodies) and feeds. The upward trend is usage of this mineralization type in paper pulp production, that is important in protection of autochthonous forests (Martínez et al. 1994), as well as in the production of biofuels, a renewable, biodegradable, and environmentally friendly energy source (Aleklett and Camphell 2003).

Trametes hirsuta (Wulf.:Fr.) Pil. synthesizes laccase and Mn-dependent peroxidase, for which the genes have already been characterized and cloned, as well as a series of cellulases and cellobiose dehydrogenase (Nakagame et al. 2006).

Wheat straw, cornstalks, mandarin orange, and orange peels represent very abundant wastes in numerous countries worldwide. However, these residues could be prospective substrates for bioconversion into fungal biomass and lignocellulolytic enzymes, due to their appropriate chemical composition (Table 1).

Table 1. Chemical Composition of the Selected Agricultural Residues

Components	Agricultural residues			
	Wheat straw (Mckean and Jacobs 1997)	Cornstalks (Oleskowicz-Popiel et al. 2008)	Mandarin orange peels (Chapman 1968; Tsiklauri et al. 1999)	Orange peels (Ververis et al. 2007)
lignin (%)	16 - 23	16.6	9 – 11	2.1
cellulose (%)	29 - 41.6	51.7	12 – 14	13.6
hemicellulose (%)	26 - 32	19.5	4 - 6	6.1
total nitrogen (%)	0.53	0.997	1.1 – 1.3	2.55
proteins (%)	3.3	7.9	0.9	1.5
Ca (ppm)	1130 - 8230	69.88	706	1610
Cu (ppm)	3 - 6	3.01	0.6	0.9
Fe (ppm)	21 - 175	27.11	2.6	8
Mn (ppm)	9.3 - 128	4.82	0.4	0
Zn (ppm)	7 - 25	55.29	0.8	2.5

The aim of the study was to evaluate the potential of *T. hirsuta* to produce ligninolytic enzymes during solid-state cultivation into the selected agricultural residues.

EXPERIMENTAL

Materials

Trametes hirsuta BEOFB 30 was collected from *Prunus* sp. in Belgrade. Culture on malt agar medium (MA) is maintained in the culture collection of the Institute of Botany, Faculty of Biology, University of Belgrade (BEOFB).

Methods

Growth conditions

The inoculum preparation comprised several steps: *(i)* inoculation of 100 mL of synthetic medium (glucose, 10.0 g/L; NH_4NO_3, 2.0 g/L; K_2HPO_4, 1.0 g/L; NaH_2PO_4 x H_2O, 0.4 g/L; $MgSO_4$ x $7H_2O$, 0.5 g/L; yeast extract, 2.0 g/L; pH 6.0) with 25 mycelial

discs (Ø 0.5 cm, from 7-day-old culture from MA); *(ii)* incubation at room temperature (22 ± 2 ºC) on a rotary shaker (160 rpm) for 7 days; *(iii)* washing of obtained biomass (3 times) by sterile distilled water (dH_2O); and *(iv)* biomass homogenization using 100 mL of sterile dH_2O in a laboratory blender.

The ligninolytic enzymes production was studied after solid-state fermentation (SSF) of selected agricultural residues: wheat straw, cornstalks, mandarin orange, and orange peels. Cultivations were carried out at 25 ºC in 100 mL flasks containing 2 g of wheat straw, or 3 g of cornstalks, or 5 g of mandarin orange, or orange peels soaked with 10 mL of the modified synthetic medium (without glucose, with nitrogen in a concentration of 10 mM, and pH 5.0). Different amounts of agricultural residues were used, considering the specific density of diverse materials. Specific density correlates with active surface available for mycelial growth. Therefore, proper approximations have been made in relation of volume and granulation, which inevitably led to different weights of agricultural residues to be used. The potential of mandarin orange or orange peels was also evaluated in glucose-enriched modified synthetic medium due to lower amount of hydrocarbons. The prepared flasks were inoculated with 3 mL of homogenized inoculum.

Samples from flasks were harvested after 7 days of cultivation and ligninolytic enzymes were extracted by stirring with 50 mL of dH_2O on magnetic stirrer for 10 min at the temperature of 4ºC. The obtained extracts were separated by centrifugation (3000 rpm, 10 min, 4 ºC), and the supernatants were further used for measurement of activity of laccase (EC 1.10.3.2), aryl alcohol oxidase (EC 1.1.3.7), lignin peroxidase (EC 1.11.1.14), and Mn-oxidizing peroxidases (EC 1.11.1.13), as well as total protein content. Three replicates for each analyzed plant residue were prepared in order to decrease statistical error.

Enzyme activity assays

Activities of selected ligninolytic enzymes were determined spectrophotometrically.

Laccase (Lac) activity was estimated by monitoring the A_{436} change related to the rate of oxidation of 50 mM 2,2'-azino-bis-[3-ethyltiazoline-6-sulfonate] (ABTS) (ε_{436} = 29300 M^{-1} cm^{-1}) in 0.1 M phosphate buffer (pH 6.0), at 35 ºC. The reaction mixture (V_{tot} = 1 mL) contained: buffer, ABTS, and sample (Stajić et al. 2006, 2010).

Aryl alcohol oxidase (AAO) level was tested as the oxidation of veratryl alcohol to veratraldehyde (ε_{310} = 9300 M^{-1} cm^{-1}). The reaction mixture (V_{tot} = 1 mL) contained 0.1 M phosphate buffer (pH 6.0), 5 mM veratryl alcohol, and sample (Camarero et al. 1996, 1999).

Lignin peroxidase (LiP) activity was also assayed by oxidation of veratryl alcohol to veratilaldehyde (ε_{310} = 9300 M^{-1} cm^{-1}) initialised by addition of 2 mM H_2O_2. The reaction mixture (V_{tot} = 1 mL) contained: 0.1 M tartarate buffer (pH 3.0), 2 mM veratryl alcohol, 2 mM H_2O_2, and sample (Camarero et al. 1999).

Activities of Mn-oxidizing peroxidases [Mn-dependent peroxidase (MnP) and versatile peroxidase (VP)] were determined with 3 mM phenol red (ε_{610} = 22000 M^{-1} cm^{-1}) as a substrate, in a buffer with the following content: succinic acid disodium salt, albumin from bovine serum, and DL-lactic acid sodium salt (pH 4.5). The reaction mixture (V_{tot} = 1 mL) contained: buffer, sample, 2 mM H_2O_2, and phenol red, with or

without 2 mM $MnSO_4$ (for MnP and VP, respectively). The reaction was stopped by 2M NaOH (Stajić et al. 2006, 2010).

Enzymatic activity of 1 U is defined as the amount of enzyme that transforms 1 μmol of substrate/min. A CECIL CE2501 Spectrophotometer was used for these assays. The specific enzyme activity presents relation of enzyme activity and the total protein content expressing as U/mg.

Determination of total proteins

The amount of total proteins was determined by the method of Bradford using bovine serum albumin as standard (Silva et al. 2005). Total protein content was defined as difference in total protein amount between sample and control and presented as mg/mL. The plant residues treated in the same way as samples but without organism were used as the controls.

Statistical analysis

The results were expressed as the mean ± standard error of data obtained from triplicate experiments. One-way analysis of variance (ANOVA) was used to test the significance of differences among the plant residues, using STATISTICA software, version 5.0 (StatSoft, Inc). P-values less then 0.01 were considered statistically significant.

RESULTS

Activities of Lac and Mn-oxidizing peroxidases were detected after 7 days of solid-state fermentation of all tested agricultural residues by *T. hirsuta* BEOBF 30 (Fig. 1A). The wastes showed the significantly different potential for stimulation of enzyme production ($P<0.01$).

The maximum of Lac activity (3827.0 ± 219.0 U/L) was obtained in the medium with mandarin orange peels as sole carbon source, while enrichment of the same medium with glucose caused its decrease (2665.0 ± 64.5 U/L) (Fig. 1A). Activity of this enzyme was lower into orange peels without and with glucose, as well as into cornstalks (2500.0 ± 122.0, 2172.0 ± 24.0, and 1803.0 ± 30.5 U/L, respectively). The wheat straw was shown to be an unfavourable substrate for Lac production, and the lowest level of Lac activity (910.0 ± 111.0 U/L) was noted in this medium.

Contrary to the Lac synthesis, wheat straw was the optimal substrate and mandarin orange peels the most unfavourable medium for MnP production (1971.5 ± 23.0 and 224.5 ± 30.0 U/L, respectively). The similar levels of MnP activity were noted in the media containing orange peels, glucose-enriched orange peels, cornstalks, as well as glucose-enriched mandarin orange peels (1329.5 ± 108.0, 1139.0 ± 90.5, 1060.0 ± 76.5, and 1057.0 ± 88.0 U/L, respectively) (Fig. 1A).

The highest value of VP activity (1173.0 ± 100.0 U/L) was obtained in glucose-enriched mandarin orange peels medium and the lowest one (403.5 ± 4.5 U/L) in the medium where mandarin orange peels were the sole carbon source. Similar levels of VP production (ranged between 707.5 ± 81.0 U/L into cornstalks medium and 829.5 ± 74.0

U/L into glucose-enriched orange peels medium) were detected in the remaining four tested media (Fig. 1A).

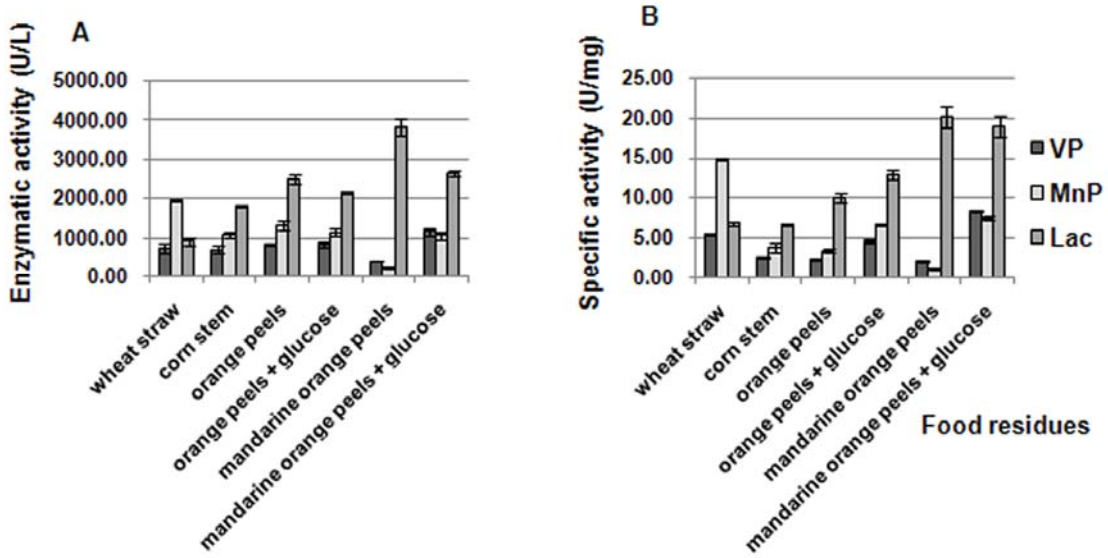

Fig. 1. Effect of selected agricultural residues on activity of Mn-oxidizing peroxidases and laccase in *Trametes hirsuta* BEOFB 30

The level of veratryl alcohol oxidation in the presence of H_2O_2 was the highest in the glucose-enriched mandarin orange peels medium (11591.5 ± 122.9 U/L) and the lowest in the wheat straw and cornstalks media (1679.6 ± 12.3 and 1822.6 ± 102.7 U/L, respectively). Values of veratryl alcohol oxidation in the absence of H_2O_2 were similar to those noted in the presence of H_2O_2 but slightly lower, for all tested substrates.

The total protein production was the highest during fermentation of cornstalks medium (13.5 ± 0.2 mg/mL) and the lowest in wheat straw containing medium (6.6 ± 0.5 mg/mL), which was reflected in the specific enzymes activities (Fig. 1B). Similar trends in activity level could be noted by comparing enzymatic activities with their specific activities (Fig. 1A, B). An exception was detected in Lac activity during orange peels fermentation, where lower specific activity in treatment without glucose (10.1 ± 0.6 U/mg) than in glucose-enriched medium (12.9 ± 0.7 U/mg) was noted.

DISCUSSION

This study showed that *T. hirsuta* has potential for fermentation of selected agricultural residues under solid-state conditions. This was achieved due, first of all, to the significant production of ligninolytic enzymes. Annual worldwide production of agricultural plants is considerable, 123 x 10^6 t, and approximately half of that amount is used neither for food and feed, nor for the production of textile and paper (Villas-Bôas et al. 2002). Therefore, these abundant and available bio-wastes could present serious environmental pollutants; however they are also good substrates for obtaining large amounts of low-cost enzymes and bioconversion into fungal biomass (Moldes et al. 2004;

Songulashvili et al. 2007). The utilization of the raw plant materials by various mushroom species could contribute to the more environmentaly friendly measures for solving pollution problems.

Production of ligninolytic enzymes varied within a wide range depending on species and strain, as well as numerous cultivation factors, such as: cultivation type (submerged or solid-state), carbon and nitrogen sources and concentrations, presence or absence of different inducers, medium pH, temperature, agitation, cultivation period, etc. (Songulashvili et al. 2007; Stajić et al. 2006, 2010; Simonić et al. 2008). Solid-state cultivation is more suitable for production of enzymes and metabolites that usually will not be synthesized or will be synthesized at low amount under submerged conditions. Reasons for that are, first of all, particle size, porosity, and moisture content close to the fungal natural habitats. In an attempt to provide equal conditions for biomass production and enzyme synthesis, glucose enrichment of citrus peels media has been carried out. Lower hydrocarbons content of orange and mandarin orange peels (16 – 20%) compared with wheat straw and cornstalks (55 – 73% and 70%, respectively), as well as the glucose role in yeast extract consumption in the initial phases of mushroom growth were crucial for the approach. Wagner et al. (2004) showed that the presence of glucose during *Ganoderma lucidum* cultivation stimulates growth in the medium with peptone and yeast extract as nitrogen sources, even during periods in which glucose is not being consumed. In the presence of glucose, its small amounts are consumed in order to produce precursors that formed in the initial part of glycolysis and pentose phosphate pathway, and therefore a much more intense growth is possible due to consuming the yeast extract and peptone.

There are reasonable assumptions that sugar and nitrogen concentrations in the residues could affect Lac activity synergistically. The obtained results indicated that there is a positive correlation between polysaccharides and nitrogen content in studied residues and Lac activity in *T. hirsuta* BEOFB 30. Significantly higher levels of Lac activity were noted after 7 days of citrus fruits peels SSF, than wheat straw and cornstalks ($P<0.01$). This could be explained by the chemical composition of these raw plant materials (Table 1). Lower content of polysaccharides and higher nitrogen amount in citrus peels stimulated the enzyme biosynthesis, which was in accordance with the data of Stajić et al. (2006), while opposite to the results of Srinivasan et al. (1995), Hammel (1997), and Master and Field (1998). Contrary, glucose-enriched mandarine orange and orange peels media caused significant decrease of Lac activity in *T. hirsuta* BEOFB 30 ($P<0.01$), which is in accordance with results of Galhaup et al. (2002) who reported that higher glucose concentrations could repress Lac synthesis and genes that take part in the metabolism of carbon sources in *T. pubescens*. However, Moldes et al. (2003) showed that maintaince of high glucose quantity in orange peels along cultivation is an essential factor to keep high level of this enzyme production by staded *T. hirsuta* strain. As a conclusion, it could be emphasized that the glucose level that suppresses Lac synthesis is different from species to species, and even from strain to strain.

Tomšovský and Homolka (2003) also noted that *T. hirsuta* was the best Lac producer among the studied *Trametes* species. Rodrígez Couto et al. (2006), Rosales et al. (2002, 2007), and Osma et al. (2007) reported that orange peels and mandarin orange peels have high potential for stimulation of Lac synthesis by *T. hirsuta* and *T. pubescens*,

respectively. In addition, citrus fruits peels, apple, potato and kiwi peels, grape seeds, coconut flesh, as well as barley and rice bran have also been shown to be good substrates for Lac production by *T. hirsuta* during solid-state cultivation (Moldes et al. 2003; Rosales et al. 2002, 2005; Rodriguez Couto et al. 2004; Rodriguez Couto and Sanroman 2005; Perumal et al. 2007).

In contrast to Lac production, MnP synthesis by *T. hirsuta* BEOFB 30 was the highest in wheat straw medium and the lowest in mandarin orange peels medium. Schlosser et al. (1997) also noted good MnP production by *T. versicolor*, and Winquist et al. (2008) by *T. hirsuta* during solid-state cultivation into wheat straw and oat husks, respectively. However, during submerged fermentation of mandarin orange peels by *T. hirsuta* and *T. versicolor*, MnP synthesis was insignificant and even absent (Songulashvili et al. 2007). Since MnP and Lac act synergistically, it is important to define substrate and cultivation conditions for simultaneously high production of both enzymes.

Glucose-enriched mandarin orange peels medium was the optimal for VP production by *T. hursuta* BEOFB 30 ($P<0.01$). VP production, as well as the relation between MnP and VP synthesis could be explained by chemical composition of the substrate (Table 1), especially by content of Mn and Ca, which have important role in the phenol compounds oxidation and stabilization of protein structure (Martinez 2002). VP synthesis shows, by the best means, dependence of enzyme production on substrate and species. Thus, Aggelis et al. (2002) also obtained a significant level of VP activity during green olives debittering wastewater fermentation by *T. hirsuta*, while Champagne and Ramsay (2005) noted the absence of its production by *T. versicolor* into wheat bran-enriched medium.

LiP has been considered as a key enzyme in lignin degradation by white rot fungi, since it is able to catalyze the H_2O_2-dependent oxidation of high redox-potential aromatic compounds including non-phenol lignin units. On the other hand, AAO is the main H_2O_2-generating enzyme involved in lignin degradation. However, the detection of their activities may be aggravating because both of them are usually assayed by oxidation of veratryl alcohol to veratraldehyde. Although Johansson and Nyman (1993) separated and characterized 16 forms of LiP, and Varela et al. (2000) found *lpo* gene in *T. versicolor*, data on production of this enzyme, the profiles of isoforms and genetic regulation in *T. hirsuta* are still debatable. Numerous studies showed that LiP production depends on the composition of culture medium and cultivation type. Contrary to Peláez et al. (1995), who did not detect LiP synthesis in *T. hirsuta* during submerged cultivation in synthetic medium, Tomšovskỳ and Homolka (2003) detected this enzyme in different enriched N-limited media.

Although Varela et al. (2000) did not detect the *aao* gene in *T. versicolor*, Peláez et al. (1995), as well as this study, reported a significant level of veratryl alcohol oxidation in the absence of H_2O_2 in *T. hirsuta* during submerged and solid-state cultivation, respectively. H_2O_2 plays an important role in lignin degradation as co-substrate of ligninolytic peroxidases (Kirk and Farrell 1987), but it could also be a competitive inhibitor depending on its concentration (Tien et al. 1986). Did 2 mM H_2O_2, as used in this study, act as a LiP substrate or as an inhibitor? Did *T. hirsuta* BEOFB 30 produce both LiP and AAO, or only one of them, and which one during SSF of selected

food residues? Given the fact that the widely applied veratryl alcohol assay has several shortcomings (Archibald 1992), adding to the list of uncertainties, it could be concluded that introduction of the *T. hirsuta* ligninolytic enzyme system requires more detailed study.

CONCLUSIONS

1. The highest level of laccase activity was noted in mandarin orange peels medium.
2. Wheat straw and glucose-enriched mandarin orange peels media were optimal for Mn-dependent peroxidase and versatile peroxidase production, respectively.
3. Levels of veratryl alcohol oxidation in the presence of H_2O_2 were similar to those in its absence during fermentation of all selected agricultural residues.

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