

## Lignocellulose Degradation by *Daedaleopsis confragosa* and *D. tricolor*

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The properties and capacities of the ligninolytic enzymes of *Daedaleopsis* spp. are still unknown. This is the first study on the effect of plant residues and period of cultivation on the properties of Mn-oxidizing peroxidases and laccases of *D. confragosa* and *D. tricolor*, as well as their ligninolytic potentials. Wheat straw was the optimal carbon source for synthesis of highly active Mn-dependent peroxidases (4126.9 U/L in *D. confragosa* and 2037.9 U/L in *D. tricolor*). However, laccases were the predominant enzymes, and the best inducer of their activity (up 16000.0 U/L) was cherry sawdust. Wheat straw was the most susceptible plant residue to the effect of the enzymes, and extent of lignin degradation was 43.3% after 14 days of fermentation with *D. tricolor*. However, *D. confragosa* was a more effective lignin degrader, as it converted even 21.3% wheat straw lignin on the 6<sup>th</sup> day of cultivation. The results of the study clearly showed that delignification extent depends on mushroom species and on the type of plant residue, which is extremely important for potential use in biotechnological processes.

**Keywords:** *Daedaleopsis* spp; Delignification; Ligninolytic enzymes; Lignocellulosic residues; Solid-state fermentation.

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### INTRODUCTION

A large amount of lignocellulosic residue is produced annually worldwide (150 billion tons), and the dominant biomass differs depending on the region (Asim *et al.* 2015; Gupta and Verma 2015). Production of these wastes is also remarkable in Serbia, where 51% of the territory contains agricultural crops and about 27% is forest (Jordanović-Vasić 2009). Adding the biomass of weeds, municipal, and food industry wastes to the quantity of agro-forestry residues, the combination represents an enormous amount of the wastes, which present serious ballast for the environment due to inadequate management, *i.e.*, deposition and usage. However, lignocellulosic materials could be important resources for food, feed, paper, chemicals, and energy production (Stajić *et al.* 2016). These biotechnological processes require pretreatment of the biomass. The treatment can be physical, chemical, physico-chemical, or biological, which is recently preferred due to significant delignification selectivity, minimal release of toxic by-products, and lower energy consumption (Sánchez 2009). White rot fungi play the main role in the biological biomass pretreatment; they synthesize ligninolytic enzymes, *i.e.*, lignin- and Mn-oxidizing peroxidases, laccases, and some auxiliary enzymes (Stajić *et al.* 2016). Because mushrooms have different levels of production of active ligninolytic enzymes, they have different potential in the various biotechnological processes. Thus, there have been recent studies of the enzyme properties and optimization of cultivation conditions for their synthesis and maximal delignification.

Species of the genus *Daedaleopsis* are insufficiently studied, even though they are known as white rot fungi (Marković 2012). *D. confragosa* (Bolton) J. Schröt and *D. tricolor* (Bull.) Bondavtsev & Singer are the most commonly researched species of the genus, especially with regard to their problematic taxonomy and medicinal potential (Rösecke and König 2000; Bernicchia *et al.* 2006). Although these species naturally occur as biotrophs or necrotrophs in numerous broadleaf species, some properties of their enzyme systems are not sufficiently clarified. Therefore, this study evaluated *D. confragosa* and *D. tricolor* for their potential to delignify their natural substrates—cherry and beech sawdust—and wheat straw, an alternative substrate.

## EXPERIMENTAL

### Materials

Fruiting bodies collected on Avala mountain (Serbia) and in Veliko Tarnovo (Bulgaria) were morphologically identified as *Daedaleopsis confragosa* and *D. tricolor*, respectively. The cultures were isolated from collected fruiting bodies and maintained on malt agar medium in culture collection of Institute of Botany, Faculty of Biology, University of Belgrade (BEOFB).

### Methods

#### *Cultivation conditions*

Inoculum was prepared as described previously (Stajić *et al.* 2010). Briefly, synthetic medium (glucose, 10.0 g/L;  $\text{NH}_4\text{NO}_3$ , 2.0 g/L;  $\text{K}_2\text{HPO}_4$ , 1.0 g/L;  $\text{NaH}_2\text{PO}_4 \times \text{H}_2\text{O}$ , 0.4 g/L;  $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ , 0.5 g/L; yeast extract, 2.0 g/L; pH 6.5) was inoculated with mycelial discs from 7-day old cultures grown on malt agar. Cultures were incubated at room temperature ( $22 \pm 2$  °C) on a rotary shaker (100 rpm) prior to washing and homogenization of the obtained biomass. Wheat straw, beech sawdust, and cherry sawdust fermentation were conducted under solid-state conditions for 6, 10, 14, 18, 22, and 26 days. Experiments were carried out in triplicate; results are expressed as mean  $\pm$  standard error.

#### *Assays of enzyme activity and total protein production*

The ligninolytic enzymes were extracted by sample mixing with 50.0 mL of distilled water ( $\text{dH}_2\text{O}$ ) by a magnetic stirrer (4 °C, 10 min). The obtained extracts were separated from plant residues and mycelial rests by centrifugation (3000 rpm, 4 °C, 10 min). The supernatants were used to measure the activity of Mn-oxidizing peroxidases and laccases, and the total protein content was measured with a spectrophotometer (BioQuest CECIL CE2501, Cambridge, UK). Phenol red ( $\epsilon_{610} = 22,000 \text{ M}^{-1} \text{ cm}^{-1}$ ) was used as substrate for defining activities of Mn-oxidizing peroxidases [Mn-dependent peroxidase (MnP, EC 1.11.1.13) and Mn-independent peroxidase (MnIP, EC 1.11.1.16)], and 2,2'-azino-bis-[3-ethylthiazoline-6-sulfonate] (ABTS) ( $\epsilon_{436} = 29,300 \text{ M}^{-1} \text{ cm}^{-1}$ ) for laccase activity monitoring (Stajić *et al.* 2010). The reaction mixture ( $V_{\text{tot}} = 1.0 \text{ mL}$ ) for measurement of laccases contained buffer, ABTS, and sample while the peroxidases one were composed of buffer, sample, 2.0 mM  $\text{H}_2\text{O}_2$ , phenol red, with or without 2.0 mM  $\text{MnSO}_4$  (for MnP and MnIP, respectively). An enzymatic activity of 1 U was defined as the amount of enzyme that transformed 1.0  $\mu\text{mol}$  of substrate per minute.

The total protein content of (mg/mL) was determined in a spectrophotometer ( $\lambda = 595 \text{ nm}$ ) in a reaction mixture of Coomassie brilliant blue G-250, using 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, and 1.0 mg/mL) and Bradford reagent (Silva *et al.* 2005).

### *Electrophoresis*

The profiles of Mn-oxidizing peroxidases and laccases of *D. confragosa* BEOFB 710 and *D. tricolor* BEOFB 720 were screened under cultivation conditions where all tested enzymes showed significant activities. A Mini IEF Cell-Model 111 (BIO-RAD, Hercules, CA, USA) was used for isoelectric focusing (IEF) and defining the isoelectric points (pI) of enzyme isoforms. IEF was carried out in 7.5% polyacrylamide gel with 5.0% ampholyte on a pH gradient from 3.0 to 10.0 using an IEF marker (pI range from 3.6 to 9.3; Sigma-Aldrich, St. Louis, MO, USA) (Ćilerdžić *et al.* 2016). According to this method, bands with Mn-oxidizing peroxidases activities were located *via* incubation of the gel in 4-Cl-1-naphtol/H<sub>2</sub>O<sub>2</sub>/potassium phosphate buffer mixture (with or without MnSO<sub>4</sub> for MnP and MnIP, respectively) at room temperature (22 ± 2 °C) until the appearance of dark-brown bands. Laccase bands were located by gel incubation in ABTS/phosphate buffer. After focusing, the gel was fixed in 12.0% trichloroacetic acid, and protein bands were detected by staining with the solution of 0.1% CBB R in fixative (methanol, acetic acid, and H<sub>2</sub>O in a 45:10:45 ratio).

### *Determination of hemicellulose, cellulose, and lignin contents*

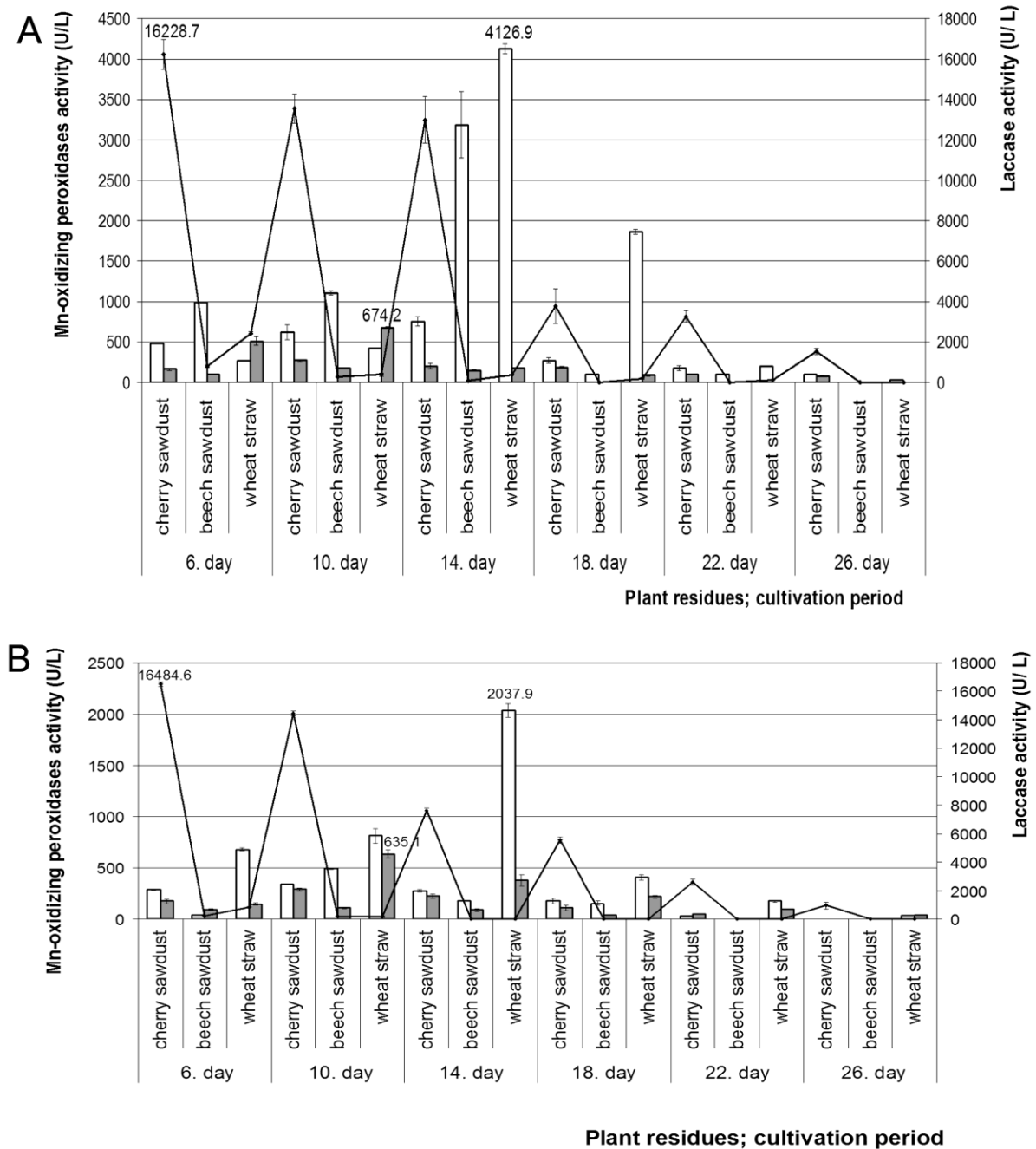
The amount of lignin, hemicellulose, and cellulose in the selected plant raw materials was defined before and after *D. confragosa* and *D. tricolor* cultivation. Hemicellulose content was determined by the method of Van Soest *et al.* (1991). To remove soluble sugars, proteins, pectin, lipids, and vitamins, a dried ground sample of 1.0 g was treated with a solution of neutral detergent and Na<sub>2</sub>SO<sub>3</sub> 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 in the tested sample was calculated as the difference between ADF and NDF.

Acidic detergent fibers were used to determine the cellulose and lignin contents. Lignin content (LC) was defined by the Klason method (Kirk and Obst 1988), by incubation of sample in 72% H<sub>2</sub>SO<sub>4</sub> at 30 °C and its hydrolysis at 120 °C. Lignin content was expressed as a percentage of that present in the original sample, while the cellulose content was calculated as ADF minus LC.

## RESULTS AND DISCUSSION

### **Enzyme Properties Depending on Plant Raw Materials and Cultivation Period**

Both *Daedaleopsis* species produced enzymes, but their activities varied remarkably depending on the carbon sources, *i.e.*, plant residue and cultivation period (Fig. 1). Wheat straw was the best carbon source for maximizing MnP activity. However, the activity depended on the cultivation period. Thus, the highest MnP activity was noted on day 14 of wheat straw fermentation by *D. confragosa* and *D. tricolor* (4126.9 U/L and 2037.9 U/L, respectively); activity decreased with prolonged cultivation to day 26 (Fig. 1). Compared with MnP, MnIP was less active, with the maximum on day 10 of wheat straw fermentation by both species (674.2 U/L *vs.* 635.1 U/L). The activity decreased gradually over the cultivation period, with almost no activity reported on day 26 (Fig. 1).

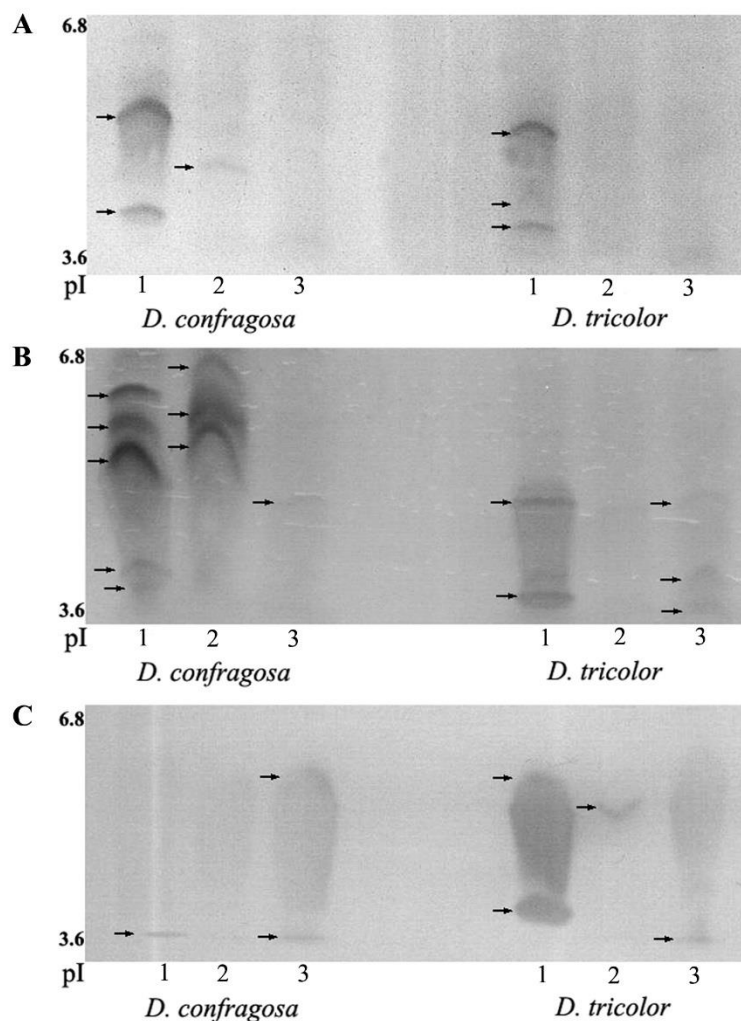


**Fig. 1.** Effect of plant residues and cultivation period on the activities of Mn-dependent peroxidase (white bar), Mn-independent peroxidase (gray bar), and laccase (-♦-) in *Daedaleopsis confragosa* (A) and *D. tricolor* (B). Data represent mean value of activities of three different samples. Variations are given as standard errors.

The optimal carbon source for laccase activity in both species was cherry sawdust, and the maximal activities, up to 16000.0 U/L, were noted on day 6 of fermentation. However, the values were also remarkable on day 10 and 14 and thereafter declined to the minimum of 949.9 U/L on day 26. Beech sawdust and wheat straw slightly stimulated the enzyme activity only on day 6 and 10 (Fig. 1). Generally, the laccase activity was significantly higher than the Mn-oxidizing peroxidase activity.

The type of plant residue and cultivation period also affected protein production and specific enzyme activities. The maximal value of specific MnP activity was noted on day 22 of cherry sawdust fermentation by *D. tricolor* (4.6 U/mg), while the maximums of MnIP (6.1 U/mg) and laccase (6.3 U/mg) were found in wheat straw fermentation by *D. confragosa* on day 18 and *D. tricolor* on day 10, respectively.

Because all tested enzymes showed remarkable high activity after 10 days of fermentation, the isoenzyme profiles were monitored at that point. The IEF profiles of Mn-oxidizing peroxidases and laccase isoforms showed variations in different plant residues. Their pI values ranged between 3.6 and 6.8, as visualized on zymograms (Fig. 2).



**Fig. 2.** Isoelectric focusing pattern of Mn-dependent peroxidases (A), Mn-independent peroxidases (B), and laccases (C) in *Daedaleopsis confragosa* and *D. tricolor* after 10-day fermentation of cherry sawdust (1), beech sawdust (2) and wheat straw (3)

In cultivation on cherry sawdust, both species produced two MnP isoenzymes of pI of about 4.6 and 5.9, while *D. tricolor* synthesized one more with a pI of 3.8. Beech sawdust induced production of only one MnP isoform (pI 5.1) in *D. confragosa*, while no isoform was detected after wheat straw fermentation by this species (Fig. 2A). The cherry sawdust was also the best inducer of MnIP isoforms (Fig. 2B). Namely, five bands at pIs 3.7, 3.8,

5.1, 5.9, and 6.6 were observed in zymograms of *D. confragosa*, while only two isoforms at pIs 3.7 and 4.6 were detected in *D. tricolor*. Likewise, beech sawdust stimulated MnIP synthesis in *D. confragosa*; several isoforms ranged from pI 3.6 to 6.8. Wheat straw was a better carbon source for the isoenzyme synthesis by *D. tricolor* (pI 3.6, 3.8, and 4.6) (Fig. 2B).

Both species produced only a few laccase isoforms during cultivation in all tested plant residues (Fig. 2C). *D. confragosa* synthesized two isoenzymes (pIs 3.6 and 5.9) during wheat straw fermentation and only one (pI 3.6) in cherry sawdust fermentation. No isoform was visualized after beech sawdust fermentation, which was different from *D. tricolor* zymograms. Namely, two bands were detected after *D. tricolor* cultivation on cherry sawdust (pI 3.8 and 5.1) and one on beech sawdust (pI 5.1) and wheat straw (pI 3.6) (Fig. 2C).

### Effect of Cultivation Conditions on Lignocellulose Degradation

The lignin, hemicellulose, and cellulose contents in the plant raw materials were different (Table 1). The extent of the polymer degradation increased during fermentation, but the rate of degradation depended on species and plant residue. However, in some cases, the depolymerization degree did not have a positive correlation with the level of enzyme activity. The tested *Daedaleopsis* species were highly effective delignifiers of wheat straw, which was the most susceptible plant residue. After 14 days of *D. confragosa* and *D. tricolor* cultivation, 43.3% and 42.4% of lignin, respectively, was degraded.

**Table 1.** *Daedaleopsis confragosa* and *D. tricolor* to Degradation of Lignocellulosic Polymers

Species	Plant Residue	Fermentation Period (days)	Extent of Polymer Degradation (%)		
			Lignin	Hemicellulose	Cellulose
<i>Daedaleopsis confragosa</i>	cherry sawdust	6	9.6	3.3	13.4
		10	16.3	11.4	13.4
		14	23.5	13.0	14.2
	beech sawdust	6	22.8	6.9	3.4
		10	29.4	8.5	12.6
		14	30.9	10.8	13.8
	wheat straw	6	21.3	15.4	2.9
		10	36.1	23.8	5.2
		14	42.4	30.3	6.3
<i>Daedaleopsis tricolor</i>	cherry sawdust	6	14.3	5.1	16.4
		10	22.8	10.1	17.3
		14	30.1	12.5	17.5
	beech sawdust	6	2.1	1.6	2.5
		10	6.0	3.0	6.3
		14	30.0	27.6	14.7
	wheat straw	6	3.9	13.6	1.4
		10	31.3	22.8	12.2
		14	43.3	23.8	13.4

The extent of cherry sawdust lignin depolymerization increased with the period of fermentation in both species, but *D. tricolor* was more of a potential degrader. Although both species mineralized almost identical amounts of beech sawdust lignin after 14 days of fermentation, *D. confragosa* showed more rapid degradation than *D. tricolor*, with 22.8% vs. 2.1% delignification, respectively, on day 6 (Table 1). *Daedaleopsis* species were better hemicellulose than cellulose mineralizers, especially *D. confragosa* that degraded even 30.3% of wheat straw hemicellulose after 14-day old fermentation. *D. tricolor* was a better degrader of cellulose, particularly after 10 days of cherry sawdust fermentation (17.3%) (Table 1). Generally, *D. confragosa* was a better degrader of wheat straw and beech sawdust polymers, while a higher extent of cherry sawdust depolymerization was obtained with *D. tricolor*.

Although studies of fungal ligninolytic enzymes are current, because of their wide biotechnological potential, data on properties and capacities of the *Daedaleopsis* spp. enzymes have remained unknown until this work. Information about the abilities of *D. confragosa* and *D. tricolor* to do ligninolysis and thus participate in transformation of lignocellulose residues to feed, paper, and energy can be considered as the main contribution of this study.

Fungal species/strains and nature of lignocellulose substrate are important factors that determine the expression and delignification activity of ligninolytic enzymes (Camarero *et al.* 1996; Elisashvili *et al.* 2008; Simonić *et al.* 2010; Arora *et al.* 2011; Knežević *et al.* 2013; Čilerdžić *et al.* 2016). There are significant differences in ligninolytic enzyme characteristics and ligninolysis capacity between the two species studied here; these differences are explained by their phylogeny and genetics. Camarero *et al.* (1996) reported interspecific diversity in MnP activity between *Coriolus hirsutus* and *C. pubescens* as well as between *Pleurotus salignus* and *P. ostreatus*, while intraspecific variabilities of laccase and Mn-oxidizing peroxidases activities were noted in *Ganoderma lucidum* by Simonić *et al.* (2010). Long-term studies also demonstrated the dependence of these enzymes' properties on plant residue composition, especially the proportion of lignin in relation to hemicellulose and cellulose (D'Souza *et al.* 1999; Fenice *et al.* 2003; Songulashvili *et al.* 2006; Čilerdžić *et al.* 2011). Songulashvili *et al.* (2006) found that substrate type was essential for MnP production and activity in *Phanerochaete chrysosporium* and *Phellinus robustus*, and Čilerdžić *et al.* (2011) showed that *Trametes hirsuta* synthesized the most active form of this enzyme during wheat straw fermentation, which was in accordance with data obtained for the studied *Daedaleopsis* species. However, activities and isoform profiles of the enzymes also depend on cultivation period due to their various metabolism (Gómez-Toribio *et al.* 2001). Namely, in *D. confragosa* and *D. tricolor*, laccases were the most active enzymes on day 6 of fermentation in all studied plant raw materials, but the highest delignification was reached on day 14 due to extraordinarily active MnPs, which attack the lignin and make it accessible for penetration by laccases.

The obtained results confirmed those of Knežević *et al.* (2013), which demonstrated differences among species in delignification ability as well as an absence of correlation with enzyme activity. Although the peak enzyme activities were reached during the early substrate colonization phase, a remarkable amount of lignin depolymerization was measured in the later phase. This result reflects that the earlier phase is associated with cell wall opening and the release of active compounds, such as reactive oxygen species, that are involved in enzyme activation and initiation of delignification; extensive lignin degradation is linked to extracellular polysaccharides (Valmaseda *et al.* 1991; Hammel *et*

al. 2002). This difference in delignification can be explained by differences in lignin composition, its ratio to hemicellulose and cellulose, as well as cell wall structural organization between grasses and wood (Li *et al.* 2012). Thus, Fukasawa *et al.* (2005) showed that *D. tricolor* strain cultivated on *Fagus crenata* removed 42.1% lignin and simultaneously mineralized 49.8% carbohydrates, while Asgher *et al.* (2016) reported an extremely high level of wheat straw delignification by *Schizophyllum commune* (72.3%).

## CONCLUSIONS

1. The properties of Mn-oxidizing peroxidases and laccase from *D. confragosa* and *D. tricolor* strains are greatly affected by the substrate type and composition and the cultivation period.
2. Laccases were the predominant enzymes.
3. Cherry sawdust was the optimal carbon source for synthesis of highly active laccases.
4. Wheat straw was the most susceptible plant residue to *Daedaleopsis* spp. enzymes.
5. *D. confragosa* was more effective lignin degrader than *D. tricolor*.

## ACKNOWLEDGEMENTS

This study was carried out under Project No. 173032, which is financially supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia.

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Article submitted: May 4, 2017; Peer review completed: July 8, 2017; Revised version received: July 27, 2017; Accepted: August 5, 2017; Published: August 16, 2017.  
DOI: 10.15376/biores.12.4.7195-7204