Зборник Матице српске за природне науке / Jour. Nat. Sci, Matica Srpska Novi Sad, № 124, 437—444, 2013

UDC 577.15
DOI: 10.2298/ZMSPN1324437K

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TRAMETES SUAVEOLENS AS LIGNINOLYTIC ENZYME PRODUCER

ABSTRACT: Species of the genus *Trametes* represent one of the most efficient lignin-degraders which can be attributed to a well developed ligninolytic enzyme system. Current trends are screening of ability of new species to produce these enzymes, as well as the optimization of conditions for their overproduction. Therefore, the aim of the study was to evaluate the potential of *T. suaveolens* to synthesize laccase and Mn-oxidizing peroxidases during fermentation of the selected plant raw materials. Level of enzyme activities was measured on 7, 10 and 14th day of submersion, as well as the solid-state fermentation of wheat straw and oak sawdust in the presence of NH_4NO_3 in previously determined optimal nitrogen concentration of 25 mM. The enzyme activity was determined spectrophotometrically using ABTS and phenol red as the substrates. The highest level of laccase activity (1087.1 U/L) was noted after 7 days of wheat straw solid-state fermentation, while during the submerged cultivation the production of the enzyme was not noted. Submerged cultivation in oak sawdust-enriched medium was the optimal for activity of Mn-dependent peroxidase (1767.7 U/L on day 14) and Mn-independent peroxidase (1113.7 U/L on day 7). Introduction of *T. suaveolens* to produce ligninolytic enzyme represented the base for further study, as well as the determination of relation between enzyme activity and rate of lignin degradation. It could lead to greater possibility of fungal species selection with high delignification capacity, which could take participation in sustainable production of food, feed, fibres, and energy, environmentally friendly pollution prevention, and bioremediation.

KEY WORDS: Laccase, Mn-oxidizing peroxidases, Plant residues, *Trametes suaveolens*

INTRODUCTION

White-rot fungi are well known for the unique set of extracellular oxidases and peroxidases that enable efficient degradation of lignin, the second (after cellulose) most abundant natural branched polymer and aromatic material on the Earth (H a m m e l, 1997; L e o n o w i c z et al., 1999). Well developed ligninolytic enzyme system in the species of the genus *Trametes*, composed from laccase and Mn-dependent peroxidase, is responsible for significant level of lignin mineralization (Ć i l e r d ž i ć et al., 2011). Regarding the fact

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that annual production of various plant raw materials is enormous (approximately 170 – 200 billion tons), the interest in their environmentally friendly transformation into valuable products such as food, feed, fibers, and biofuels where fungal ligninolytic enzymes have the main role, has increased (K i m and D a l e, 2004). Thus, wheat straw and oak sawdust represent abundant plant residues, which could be prospective substrates for the bioconversion into fungal biomass and lignocellulolytic enzymes due to their appropriate chemical composition (M c k e a n and J a c o b s, 1997; R a k i c et al., 2006). However, since efficiency of the lignin degradation depends on the potential of the organism-degrader, oxidative mechanisms, and culture conditions (W a n et al., 2010), screening and selection of new species with tremendous synthesis of the ligninolytic enzymes, as well as the defining of optimum conditions for their overproduction, has currently become a trend.

The aim of this study was assessment of the potential of *T. suaveolens* to produce ligninolytic enzymes during solid-state and submerged fermentation of selected plant raw materials.

MATERIALS AND METHODS

Organism and growth conditions

Culture of *T. suaveolens* HAI 300 was obtained from the Institute of Evolution, University of Haifa, Israel (HAI) and kept in the culture collection of the Institute of Botany, Faculty of Biology, University of Belgrade.

The inoculum was prepared by inoculation of 100 mL synthetic medium (glucose, 10.0 g/L; NH_4NO_3, 2.0 g/L; K_2HPO_4, 1.0 g/L; $NaH_2PO_4 \times H_2O$, 0.4 g/L; $MgSO_4 \times 7H_2O$, 0.5 g/L; yeast extract, 2.0 g/L; pH 6.5) with 25 mycelial disks (Ø 0.5 cm, from 7 day-old culture from malt agar), and incubation on a rotary shaker at 100 rpm, at room temperature (22 ± 2 °C) for 7 days. The obtained biomass was washed and homogenized with 100 mL of sterile distilled water in laboratory blender.

Activities of laccase and Mn-oxidizing peroxidases were studied after both solid-state and submerged fermentation of wheat straw and oak sawdust, residues that were previously dried at 50 °C and grounded to 0.5-1.0 cm.

Solid-state cultivation was carried out at 25 ºC in 100-mL flasks containing 2 g of wheat straw and 5 g of oak sawdust, respectively, as carbon sources, and 10 mL of modified synthetic medium (without glucose, with NH_4NO_3 and previously determined optimal nitrogen concentration of 25 mM, and pH 6.5). Prepared flasks were inoculated with 3 mL of homogenized inoculum. Extraction of the synthetized laccase (EC 1.10.3.2), Mn-dependent peroxidase (MnP, EC 1.11.1.13), and Mn-independent peroxidase (MnIP, EC 1.11.1.16) was performed after 7, 10, and 14 days of cultivation by stirring the samples with 50 mL of distilled water on magnetic stirrer for 10 min at the temperature of 4 ºC and centrifugation of 3000 rpm.

Fifty mL of modified synthetic medium was enriched with the same carbon sources and amounts, and it was inoculated with 5 mL of inoculum (in 250-mL flasks) and incubated at room temperature on a rotary shaker (at 100 rpm) for 7, 10, and 14 days during the submerged cultivation. The obtained biomasses were separated by centrifugation and supernatants were used to estimate the enzymatic activity.

Five replicates for each carbon source and measurement points were done.

Enzyme activity assays

Activity of the ligninolytic enzymes was determined spectrophotometrically. Laccase 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. Mn-oxidizing peroxidases activities were determined with 3 mM phenol red (ε_{610} = 22000 $M^{-1}cm^{-1}$) in succinic acid disodium salt/albumin from bovine serum/DL-lactic acid sodium salt buffer (pH 4.5). The reaction mixture (V_{tot} = 1 mL) consisted of buffer, sample, 2 mM H_2O_2, and phenol red, with or without 2 mM $MnSO_4$ (for MnP and MnIP, respectively), and reaction was stopped by 2M NaOH. Enzymatic activity of 1 U was defined as the amount of enzyme that transformed 1 μM of substrate per min.

Specific enzyme activity was defined by determination of total protein amount (mg/mL) using the method of S i l v a et al. (2005).

RESULTS

The activities of Lac and Mn-oxidizing peroxidases in crude extracts of *T. suaveolens* varied depending on the selected plant raw materials, type of fermentation, and cultivation period.

The highest MnP activity level (1767.7 ± 175.7 U/L) was recorded after 14 days of oak sawdust submerged fermentation, while the maximum MnIP activity (1113.7 ± 94.7 U/L) was obtained under the same cultivation conditions but on the 7th day (Fig. 1). Contrary to the mentioned results, the obtained values of Mn-oxidizing peroxidases activity were significantly lower in wheat straw submerged fermentation. During solid-state fermentation, wheat straw proved to be a better carbon source for MnP, and oak sawdust for MnIP production (Fig. 1).

Laccase synthesis was reported only during the solid-state cultivation in wheat straw-enriched medium, while under other studied conditions no activity of the enzyme was noted. Activity of the enzyme decreased with respect to the period of cultivation; the highest value was obtained after 7 days of cultivation (1087.1 ± 143.9 U/L), while on the 10th and 14th day the values were twice as low (495.5 ± 73.8 U/L) and even 5-fold (216.2 ± 46.5 U/L), respectivelly.

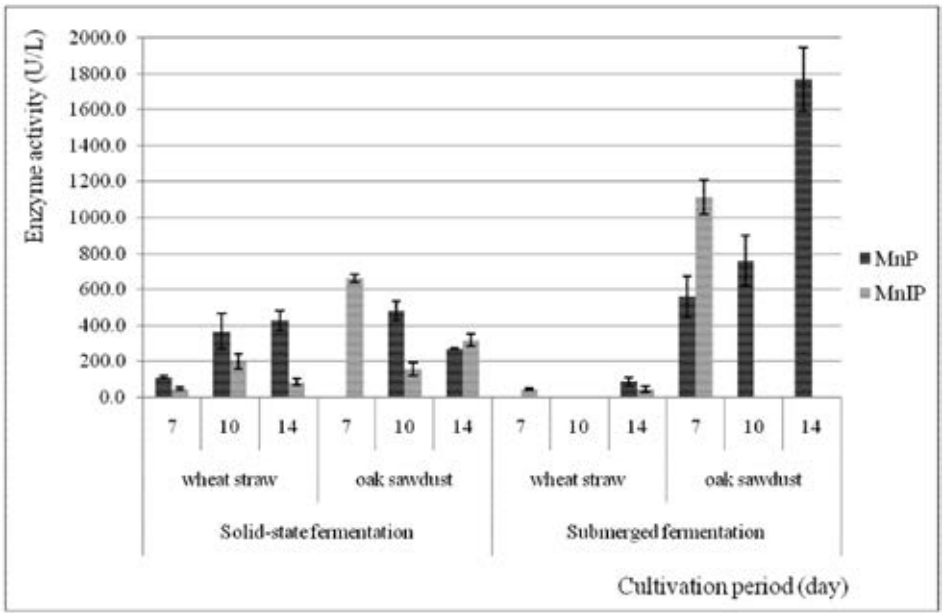

Fig. 1. – Mn-dependant peroxidase (■) and Mn-independent peroxidase (■) activity of *Trametes suaveolens* on selected plant raw materials during solid-state and submerged fermentation. The values in the figures correspond to mean values ± S.E of five replicates.

The laccase/MnP ratio changed from approximately 10:1, on the 7th day of cultivation, to 1:2, after 14 days of solid-state fermentation of wheat straw. The ratio laccase and MnIP ranged from 23:1, on the 7th day, to approximately 2.5:1 at other measurement points.

The total protein production was the highest on the 14th day of solid-state fermentation of wheat straw (147.3 ± 8.4 mg/mL) and the lowest after 14 days of solid-state cultivation in oak sawdust-enriched medium (5.7 ± 2.5 mg/mL), which was reflected on the specific enzyme activities.

DISCUSSION

The importance of this study lies in the facts the one more good lignino-lytic enzyme producer, *T. suaveolens*, was discovered, the list of well lignin degraders was broadened, and cultivation conditions for enzyme overproduction were created. E l i s a s h v i l i et al. (2008; 2009) emphasized that the enzyme synthetic potential significantly depended on the cultivation type. Namely, contrary to the results obtained for *T. suaveolens* HAI 300, results of numerous studies showed that conditions of submerged cultivation were favorable for laccase production, while solid-state cultivation was appropriate for MnP synthesis (S u n et al., 2001; J a s z e k et al., 2006; D i n i s et al.,

2009; E l i s a s h v i l i et al., 2009). Thus, S u n et al. (2001) obtained significantly higher values of MnP activity in *T. gallica*, *T. pubescens*, and *T. versicolor* when stationary submerged fermentation of wheat straw, banana and apple peelings was substituted with a solid-state one. J a s z e k et al. (2006) and D i n i s et al. (2009) also reported that MnP was the predominant ligninolytic enzyme with maximum activity being even 10 times higher than the maximum laccase level, during solid-state fermentation of wheat straw.

Contrary to the data of D i n i s et al. (2009) and A s g h e r at al. (2010), the results obtained for *T. suaveolens* HAI 300 showed that laccase and Mn-oxidizing peroxidases were not expressed constitutively and did not show synergistic action. Likewise, the maximum values of laccase and Mn-oxidizing peroxidase activity, noted on the 7^{th} and 14^{th} day of cultivation, respectively, were not in accordance with previously described mechanism of lignin biodegradation (G ó m e z – T o r i b i o et al., 2001). Namely, according to these authors, Mn-oxidizing peroxidases that oxidizes Mn^{2+} to Mn^{3+}, which then directly oxidizes lignin, are involved in initial attack on lignocellulose because of the fact that laccase is too large molecule to penetrate into non-modified plant cell walls.

Numerous studies have also demonstrated that lignocellulosic waste composition (concentrations of soluble carbohydrates and inducers), as well as species and strain physiological diversity caused by genetic variability, also affect enzyme synthesis (E r d e n e t al., 2009). E l i s a s h v i l i et al. (2008) noted significantly lower laccase/MnP ratio after wheat straw submerged fermentation by *T. versicolor* in comparison to the ratio obtained during solid-state fermentation of the same residue by *T. suaveolens* HAI 300, which could be explained by intergenetic diversity, different conditions, and availability of compounds that enhance enzyme synthesis in substrates (E l i s a s h v i l i et al., 2009).

In comparison to the other species of the genus *Trametes*, *T. suaveolens* HAI 300 belongs to a group of white-rot fungi which ligninolytic enzymes are characterized with significant activities. Thus, level of laccase activity in *T. suaveolens* HAI 300 was higher for approximately 8-fold and even 200-fold than in *T. versicolor* and *T. hirsute*, respectively, while differences in MnP activity were lower, from insignificant 2-fold to 56-fold in comparison with *T. trogii*, *T. versicolor*, and *T. hirsute*, respectively (E r d e n et al., 2009; L e v i n et al., 2010). These results could be explained by intergenetic diversity caused by evolution and adaptation to various environmental conditions.

Despite the presented data on *T. suaveolens* HAI 300 ligninolytic enzyme activities, additional research is needed to complete the knowledge about their efficiency. Namely, finding a direct correlation between enzyme activity and lignin degradation is not easy due to the complexity and variety of lignocellulose degradation mechanisms.

CONCLUSION

The obtained results showed that cultivation type and period, as well as the type of plant residue played a significant role in the regulation of ligninolytic enzyme activities in the studied species. The special contribution of the study is introduction to the *T. suaveolens* enzyme system, which has not been evaluated until now. However, despite the fact that the species represented a good enzyme producer, the synthetisis mechanism was not common when compared to the previous results; solid-state cultivation was the optimum for laccase activity while Mn-oxidizing peroxidases reached the peak of activity during submerged cultivation. A special feature of the species is the period of appearance of enzyme activity peak, which was the 7th day of cultivation for laccase and 14th day of cultivation for MnP.

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TRAMETES SUAVEOLENS ПРОДУЦЕНТ ЛИГНИНОЛИТИЧКИХ ЕНЗИМА

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РЕЗИМЕ

Врсте рода *Trametes* представљају најефикасније лигнин-деградере због добро развијеног лигнинолитичког ензимског система који чине лаказа и Mn-зависна пероксидаза. Садашњи трендови су скрининг способности нових врста да продукују ове ензиме као и оптимизација услова за њихову обимну синтезу. Због тога, циљ овог истраживања је био проучавање потенцијала *T. suaveolens* да синтетише лаказе и Mn-оксидујуће пероксидазе током ферментације одабраног биљног материјала. Ниво ензимске активности је мерен 7-ог, 10-ог и 14-ог дана течне и чврсте ферментације пшеничне сламе и пиљевине храста у присуству NH_4NO_3, у оптималној концентрацији азота од 25 mM. Ензимска активност је одређивана спектрофотометријски коришћењем ABTS и фенол црвеног као супстрата. Највиши ниво активности лаказе (1087.1 U/L) забележен је након 7 дана чврсте фер-

ментације пшеничне сламе, док у условима течне ферментације овај ензим није продукован. Течна култивација у медијуму са пиљевином храста је била оптимална за активност Mn-зависне пероксидазе (1767.7 U/L 14-ог дана) и Mn-независне пероксидазе (1113.7 U/L 7-ог дана). Познавање потенцијала *T. suaveolens* да продукује лигнинолитичке ензиме представља основу за даља истраживања, одређивање односа између ензимске активности и степена разградње лигнина. То ће водити већој могућности селекције врста са високим капацитетом делигнификације, које се могу користити у производњи хране, хранива, папира и енергије, као и у заштити животне средине и биоремедијацији.

КЉУЧНЕ РЕЧИ: лаказа, Mn-оксидујућа пероксидаза, биљни преостаци, *Trametes suaveolens*