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## EFFECT OF CULTIVATION CONDITIONS ON LIGNINOLYTIC ENZYME PRODUCTION BY *GANODERMA CARNOSUM*

**ABSTRACT:** *Ganoderma carnosum* has been found in Europe only, at coniferous trees and it is difficult to distinguish it morphologically from *G. lucidum*. Since *G. carnosum* has not been studied biochemically yet, the aim of this study was to analyse its ability to produce Mn-dependent peroxidase (MnP), versatile peroxidase (VP) and laccase (Lac) under conditions of solid-state fermentation of selected plant raw materials as carbon sources, in the presence of different nitrogen concentrations in the medium. Wheat straw, corn stem, oak and grapevine sawdust were the analysed plant raw materials. Nitrogen source in synthetic medium was  $\text{NH}_4\text{NO}_3$  and its concentrations were: 10mM N and 20 mM N. Enzyme activity was determined spectrophotometrically, using ABTS and phenol red, as the substrates for Lac and Mn-oxidizing peroxidases, respectively. Maximum level of MnP activity (56.82 U/l) was obtained in the medium with wheat straw and nitrogen concentration of 10 mM. Best carbon source for VP production was grapevine sawdust at nitrogen concentration of 10 mM (80.80 U/l). The obtained Lac activity was very low in the medium with wheat straw (1.80 U/l), while it was not detected in the presence of other three analyzed plant raw materials. Maximum of total protein content (0.06 mgml<sup>-1</sup>) was noted in the medium where oak sawdust was carbon source and nitrogen concentration was 20 mM.

**KEY WORDS:** *Ganoderma carnosum*, laccase, Mn-dependent peroxidase, nitrogen concentration, plant raw materials, versatile peroxidase

## INTRODUCTION

White-rot fungi are capable of degrading all basic wood polymers, due to their ability to synthesise relevant hydrolytic and oxidative extracellular enzymes. These enzymes are responsible for the degradation of cellulose, hemicellulose and lignin into low-molecular-weight compounds that can be assimilated for fungi nutrition (Songulashvili et al., 2007). Due to their low substrate specificity, ligninolytic enzymes can oxidize a wide range of compounds

with structural similarities to lignin, so they have important role in bioremediation of various toxic compounds in soil and waste waters (Winqvist et al., 2008). The enzymes produced by *Ganoderma* species as white-rot ones are: lignin peroxidases (LiP), Mn-oxidizing peroxidases (Mn-dependent peroxidases (MnP) and versatile peroxidases (VP)), and laccases (Lac). Contrary to *G. lucidum*, which ligninolytic system is extensively studied during the last few decades, the other species of this genus are not adequately investigated. *Ganoderma carnosum* Pat. (syn. *G. atkinsonii* Jahn, Kotl. and Pouz.) has only been found in Europe only and it is difficult to distinguish it morphologically from *G. lucidum*. This species belongs to the *G. valesiacum* complex together with *G. oregonense*, *G. tsugae* and *G. valesiacum*. This group is apparently restricted to coniferous forests in the Northern Hemisphere (Moncalvo et al., 1995). *G. lucidum*, a white-rot Basidiomycetes is widely distributed worldwide and grows predominantly on deciduous trees (*Quercus*, *Acer*, *Alnus*, *Betula* etc.) and rarely on coniferous trees (*Larix*, *Picea*, *Pinus*) (Wasser and Weis, 1997). In contrast to *G. lucidum*, this species has not been studied biochemically yet (Keller et al., 1997). Therefore, the aim of this study was to research the effect of different plant raw materials used as carbon sources, and different nitrogen concentrations on the enzyme production by *G. carnosum* under the conditions of solid-state cultivation.

## MATERIAL AND METHODS

### *Organism and growth conditions*

*Ganoderma carnosum*, collected from conifer tree in Rožaje (Montenegro), was used in this study. The culture was preserved on malt agar medium, in the culture collection of the Institute of Botany, Faculty of Biology, University of Belgrade (Serbia).

The inoculum was prepared by inoculation of 100 ml of synthetic medium (glucose, 10.0 g l<sup>-1</sup>; NH<sub>4</sub>NO<sub>3</sub>, 2.0 g l<sup>-1</sup>; K<sub>2</sub>HPO<sub>4</sub>, 1.0 g l<sup>-1</sup>; NaH<sub>2</sub>PO<sub>4</sub> x H<sub>2</sub>O, 0.4 g l<sup>-1</sup>; MgSO<sub>4</sub> x 7H<sub>2</sub>O, 0.5 g l<sup>-1</sup>; yeast extract, 2.0 g l<sup>-1</sup>; pH 6.5) with 25 agar discs (Ø 0.5 cm) taken from 7 day-old *G. carnosum* culture. Incubation was performed at room temperature (22±2°C), on a rotary shaker (160 rpm), during 7 days. The obtained biomass was washed 3 times by sterile distilled water (dH<sub>2</sub>O) and homogenized with 100 ml of sterile dH<sub>2</sub>O in a laboratory blender.

### *Effect of different plant raw materials as carbon sources and nitrogen concentrations on the production of laccase and Mn-oxidizing peroxidases*

Analyzed plant raw materials were: wheat straw, corn stem, oak and grapevine sawdust. Solid-state fermentation was carried out at 25°C in 100 ml flasks containing 2g of analysed plant residue soaked with 10 ml of the modi-

fied synthetic medium, without glucose, and with one of the two tested concentrations of nitrogen (10 mM and 20 mM) in the form of  $\text{NH}_4\text{NO}_3$  and pH 5.0. Thus, the prepared flasks were inoculated with 3 ml of homogenized inoculum. Samples from flasks were harvested after 7 days of cultivation, and extracellular enzymes were extracted by stirring of samples with 50 ml of  $\text{dH}_2\text{O}$  on a magnetic stirrer for 10 min at temperature of  $4^\circ\text{C}$ . The obtained extracts were separated by centrifugation ( $4^\circ\text{C}$ , 3000 rpm, 15 min), and the obtained supernatants were further used for measurements of the Lac, MnP and VP activity, as well as total protein content. Three replications for each analysed plant residue and nitrogen concentration were prepared, in order to decrease statistical error.

### *Enzyme activity assays*

Laccase activity was assayed spectrophotometrically, using 50 mM ABTS ( $\epsilon_{436} = 29300 \text{ M}^{-1}\text{cm}^{-1}$ ) as a substrate, in a phosphate buffer (pH 6.0). The reaction mixture contained: buffer, ABTS, and sample ( $V_{\text{tot}} = 1 \text{ ml}$ ).

Mn-oxidizing peroxidases activities were determined with 3 mM phenol red ( $\epsilon_{610} = 22000 \text{ M}^{-1}\text{cm}^{-1}$ ) as a substrate, in a buffer with the following contents: succinic acid disodium salt, albumin from bovine serum, and DL-lactic acid sodium salt, pH 4.5. The reaction mixture contained: buffer, sample, 2 mM  $\text{H}_2\text{O}_2$ , and phenol red, with or without 2 mM  $\text{MnSO}_4$ , for MnP and VP, respectively ( $V_{\text{tot}} = 1 \text{ ml}$ ). Reaction was stopped by adding 2 M NaOH. Enzymatic activity of 1 U is defined as the amount of enzyme that transforms 1 mmol of substrate per minute. An UV-160 A Spectrophotometer (Shimaden) was used for these assays.

### *Determination of total proteins*

The amount of total proteins was determined by means of a standard curve obtained from solutions containing bovine serum albumin at known concentrations (0.00; 0.01; 0.02; 0.03; 0.04; 0.05; 0.06; 0.07;  $\text{mgml}^{-1}$ ), Bradford's reagent (0.2 ml), and sufficient water to complete a final volume of 1 ml. The mixture contained 0.80 ml of the sample and 0.20 ml of Bradford's reagent, and absorbance was measured at 595 nm after reaction for 5 minutes, at room temperature. Total protein content is given in  $\text{mgml}^{-1}$ .

## RESULTS AND DISCUSSION

After 7 days of solid-state cultivation of *G. carnosum*, the activity of MnP and VP was detected in the media with all tested plant raw materials and both analysed nitrogen concentrations. In contrast to them, Lac activity was detected only in the medium with wheat straw as a carbon source (Fig. 1).

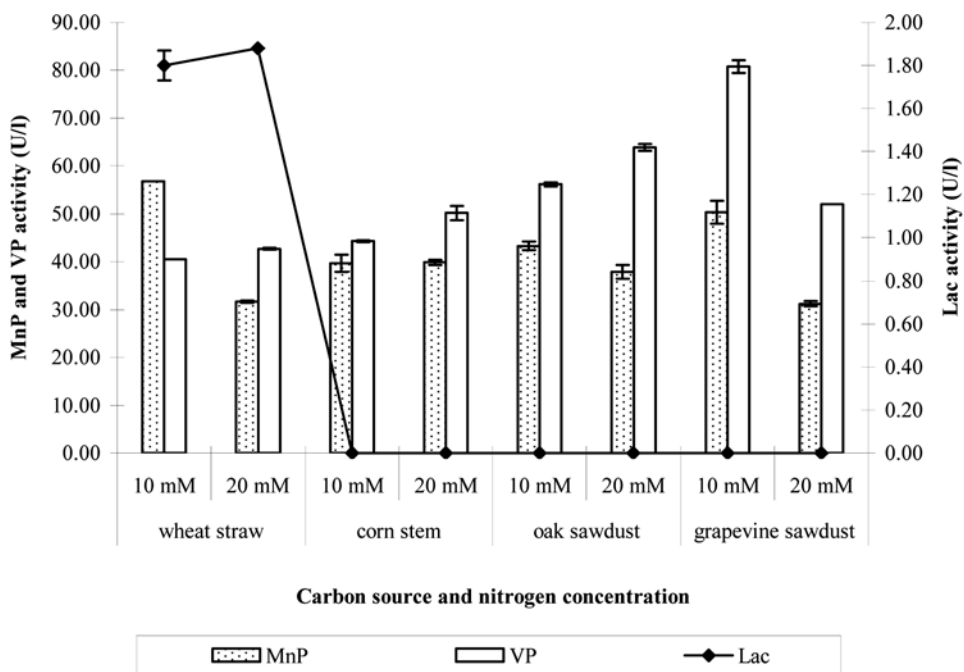


Fig. 1 — MnP, VP and Lac production depending on carbon source and nitrogen concentration

The maximum of MnP activity (56.82 U/l) was obtained in the medium with wheat straw and nitrogen concentration of 10 mM, which is in accordance with the results of Camarero et al. (1996). An increase in the nitrogen concentration to 20 mM has led to a decrease of MnP production (31.64 U/l) (Fig. 1). This can be explained by the fact that nitrogen limitation in the medium is trigger for ligninolytic enzyme production (Hamme1, 1997). Numerous studies have also shown that high nitrogen levels repressed ligninolytic enzyme production in *Phanerochaete chrysosporium*, *Trametes versicolor* and *Pycnosporus cinnabarinus* (Buswell et al., 1984; Eriksson et al., 1990; Tekere et al., 2001). However, in *Pleurotus ostreatus* high concentration of nitrogen in the medium did not repress but slightly stimulated mineralization of lignin, as compared to the nitrogen — limited medium (Kaal et al., 1995). Similar, but slightly lower value for MnP activity was detected in the medium with grapevine sawdust, at nitrogen concentration of 10 mM (50.34 U/l), while at nitrogen concentration of 20 mM MnP activity was significantly lower (31.18 U/l). Grapevine sawdust was also good substrate for Mn-oxidizing peroxidases production by *P. ostreatus* and *P. pulmonarius* (Stajić et al., 2006). The level of MnP production was lower in the media with corn stem and oak sawdust as carbon sources than in the two afore mentioned carbon sources. The measured values were similar in these media and ranged between 37.90 and 43.30 U/l (Fig. 1).

The VP activity profile showed that optimal carbon source was grapevine sawdust with nitrogen concentration of 10 mM (80.80 U/l). This is in accordance with some earlier results (Stajić et al., 2006) which showed that grapevine sawdust is good substrate for VP production by *P. ostreatus* and *P. pulmonarius*. Production of VP has decreased with increasing the nitrogen concentration to 20 mM (52.04 U/l). Relatively high values for VP production were detected in the medium with oak sawdust as carbon source and both nitrogen concentrations (56.19 U/l and 63.86 U/l at the nitrogen concentration of 10 mM and 20 mM, respectively). Slightly lower VP activity was noted in the medium with corn stem as carbon source, but the minimum of VP production (40.45 U/l) was obtained in the medium with wheat straw, at the nitrogen concentration of 10 mM, contrary to MnP production which had the peak of activity in this medium. One more difference observed in relation to MnP production is that higher nitrogen concentration has led to an increased VP production, in all analysed media, except the medium with grapevine sawdust.

Laccase production was detected only in the medium with wheat straw as carbon source, and obtained activities were similar at both nitrogen concentrations (1.80 U/l at 10 mM N and 1.88 U/l at 20 mM N) (Fig. 1). These results show that either the cultivation conditions or the selected species were reason for low level of Lac production (Stajić et al., 2009).

Maximum of total protein content of 0.060 mgml<sup>-1</sup> was detected in the medium with oak sawdust and nitrogen concentration of 20 mM, and the mini-

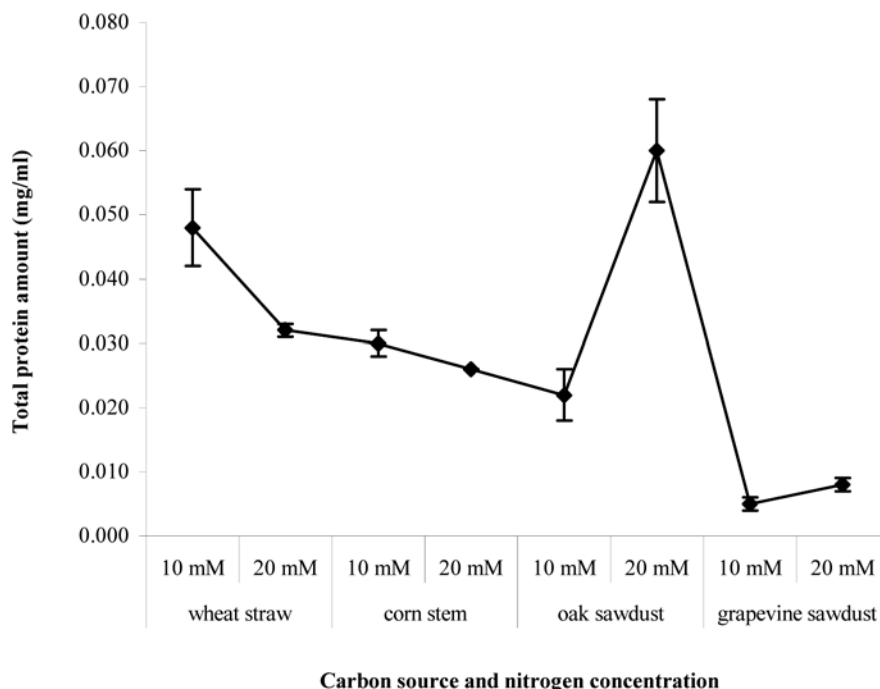


Fig. 2 — Effect of selected plant raw materials and nitrogen concentrations on total protein content

mum one of 0.005 mgml<sup>-1</sup> is measured in medium with grapevine sawdust and nitrogen concentration of 10 mM (Fig. 2).

## CONCLUSION

According to the presented results, it can be concluded that different plant residues used as carbon sources, as well as nitrogen concentrations, considerably affect the production of MnP, VP and Lac and total protein content by *G. carnosum*, during solid-state cultivation. Moreover, *G. carnosum* is a far weaker producer of ligninolytic enzymes than *G. lucidum*.

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#### УТИЦАЈ УСЛОВА КУЛТИВАЦИЈЕ НА ПРОДУКЦИЈУ ЛИГНИНОЛИТИЧКИХ ЕНЗИМА КОД *GANODERMA CARNOSUM*

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#### Резиме

Врсте рода *Ganoderma* су продуценти многих биолошки активних супстанци па су објекти проучавања многих медицинских и фармацеутских студија. У новије време се све више пажње поклања упознавању њиховог лигнинолитичког ензимског система, у циљу успешне примене у различитим биотехнолошким процесима. За разлику од *G. lucidum*, чији је лигнинолитички систем интензивно проучаван, остале врсте овог рода су ретко биле објекти истраживања. *G. carnosum* се тешко морфолошки разликује од *G. lucidum*. и још увек није биохемијски проучена, па је циљ нашег истраживања био анализа продукције Mn-оксидујућих пероксидаза и лаказа у условима чврсте култивације, на различитим биљним остацима (пшенична слама, стабљике кукуруза, пиљевина храста и винове лозе) у присуству азота у облику  $\text{NH}_4\text{NO}_3$  и у концентрацијама од 10 mM, односно 20 mM. Максимум продукције Mn-зависне пероксидазе добијен је на пшеничној слами при концентрацији азота од 10 mM (56.82 U/l). Пиљевина винове лозе и концентрација азота од 10 mM били су оптимални за синтезу верзатил пероксидазе (80.80 U/l). Добијена активност лаказа је била изузетно ниска у медијуму са пшеничном сламом (1.80 U/l), док на осталим биљним остацима није забележена. Максимални садржај укупних протеина је био највећи у медијуму са пиљевином храста као извором угљеника и концентрацијом азота од 10 mM.