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REGULAR ARTICLE

DETERMINATION OF ENZYMES PRODUCED BY *CERIPORIOPSIS SUBVERMISPORA* DURING PRETREATMENT OF DIFFERENT BIOMASS SOURCES

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ABSTRACT

The aim of this paper was to study of lignocellulolytic enzymes producing by *Ceriporiopsis subvermispora* during its cultivation on three types of plant biomass differentiated by chemical composition and physical properties (wheat straw, pine and poplar wood). The activity of lignocellulolytic enzymes in cultivation medium was determined by catalytic transformation of their natural substrates to products which were detected by photometric methods. Cellulase activities were very low while xylanases predominated. Wheat straw was best substrate for production of cellulases (4.38 U/mL) and xylanases (23.34 U/mL). The maximum activity of cellulase and xylanase was reached at 8th and 3rd day, respectively. Laccase activity reached the maximum after 16 days and then gradually decreased. The best substrate for production of laccases was poplar wood (1.67 U/mL).

Keywords: *Ceriporiopsis subvermispora,* white – rot fungus, biomass, laccases, hydrolytic enzymes

INTRODUCTION

In the nature, two types of white rot decay are observed: (i) the simultaneous degradation of all polymers in wood (cellulose, hemicelluloses and lignin) and (ii) selective degradation of lignin. *Ceriporiopsis subvermispora* is one of the best a selective lignin decomposer **(Akhtar *et al.*, 1998; Souza – Cruz *et al.*, 2004; Tanaka *et al.*, 2009)**. The white-rot fungus *C. subvermispora* has been selected for biopulping processes because it grows on wood aggressively and primary degrades lignin, therefore is suitable for the biotreatment of both soft and hardwoods **(Akhtar *et al.*, 1998)**.

C. subvermispora produces oxidoreductases and hydrolases during wood biodegradation. *C. subvermispora* lacks lignin peroxidase (LiP) activity, although LiP-like genes have been detected **(Rajakumar *et al.*, 1996)**. It produces laccase and manganese peroxidases (MnP), as well as hemicellulases and the lack of complete cellulose – degrading system **(Guerra *et al.*, 2003; Souza – Cruz *et al.*, 2004)**. From ligninolytic enzymes, laccases were described as the single predominant enzymes that were capable of lignin degradation (**Eggert et al., 1998**). Laccases degrade phenol subunits and non-phenolic lignin constituents together with mediators **(Pozdnyakova et al., 2004)**.

The aim of this work was to study of lignocellulolytic enzymes producing by *Ceriporiopsis subvermispora* during its cultivation on three types of plant biomass differentiated by chemical composition and physical properties (wheat straw, pine and poplar wood).

MATERIAL AND METHODS

Microorganism

Culture of *Ceriporiopsis subvermispora* ATTC 90467 was provided from the Centraalbureau voor Schimmelcultures (Utrecht, The Netherlands). The culture was maintained on malt agar and stored at 4 °C. In all cases, the suspension of fungal mycelium was prepared by scraping of plaque (3 cm^2) of the growth culture from agar plate using microbiological loop in sterile deionised water (10 mL). For inoculation of 100 mL of cultivation medium, 10 mL of fungal mycelium suspension was used.

Source of biomass

The biomass sources wheat straw (*Triticum aestivum*), soft wood of pine (*Pinus nigra*) and hard wood of poplar (*Populus alba*) were obtained from region Male Karpaty, Slovak Republic. These were homogenized to the size less as 0.5 cm and sterilized at 121 °C during 20 min in mineral medium with peptone as nitrogen source.

Medium composition

The composition of basic mineral medium is shown in Table 1. 50 mL of liquid medium containing glucose as carbon source (10 g/L) and peptone (5 g/L) was inoculated with 5 mL fungal mycelium suspension. This liquid culture was maintained shaken (min. 200 RPM) for 7 days at 30 °C.

Table 1 The composition of basic mineral medium

Components of medium	Concentration
$MgSO_4 . 7 H_2O$	0.5 g/L
NaCl	0.1 g/L
$CaCl_2 . 2 H_2O$	0.1 g/L
$CuSO_4 . 5 H_2O$	0.1 mg/L
$FeSO_4 . 7 H_2O$	0.2 mg/L
$MnSO_4 . H_2O$	0.02 mg/L
$ZnCl_2$	0.15 mg/L

Thereafter, liquid medium was decanted and the grown biomass of *C. subvermispora* was used to inoculate the liquid medium with peptone as nitrogen source (5 g/L) and three different source of substrates (wheat straw, pine wood and poplar wood) in the concentration 5 g/L. The enzyme (cellulases, xylanase, laccase) activities in the supernatant of medium during the cultivation of *C. subvermispora* were analysed in the intervals of 0, 1, 2, 3, 8, 10, 14, 20 and 22 days.

Enzymes assays

Cellulases

Cellulases were assayed with α-cellulose as the substrate **(Bailey *et al.*, 1992)**. The reaction mixture for cellulase activity determination contained 1.0 mL of McIlvain buffer (pH 4.8) with 50 mg α-cellulose and 0.5 mL extract of enzymes. The reaction mixture was incubated for 24 hours at 30 °C. Subsequently, the reaction mixture was centrifuged and the supernatant was assayed. The released reducing sugars were determined by 3,5-dinitrosalicylic acid. One unit (U) of enzyme activity was defined as the amount of enzyme required to liberate 1 mmol of product per min. Enzyme yield was expressed as U/mL.

Xylanase

Xylanase was assayed with xylan as the substrate **(Bailey *et al.*, 1992)**. The reaction mixture for xylanase activity determination contained 1.0 mL of McIlvain buffer (pH 4.8) with 50 mg xylan and 0.5 mL extract of enzymes. The reaction mixture was incubated for 24 hours at 30 °C. Subsequently, the reaction mixture was centrifuged and the supernatant was assayed. Released reducing sugars were determined by 3,5-dinitrosalicylic acid. One unit (U) of enzyme activity was defined as the amount of enzyme required to liberate 1 mmol of product per min. Enzyme yield was expressed as U/mL.

Laccases

Laccase activity was determined by the oxidation of catechol **(Evans and Palmer, 1983)**. Products of oxidation were measured photometrically at 465 nm. The reaction mixture contained 1.5 mL of 0.1 M phosphate buffer (pH 6.0) with 40 mM catechol and 0.5 mL of enzyme extracts. One unit of enzyme activity (U) was defined based on the comparison of the standard preparation of laccase (Sigma, Germany, isolated from *Trametes versicolor*) possessing the activity 21.8 U/mg. Enzyme yield was expressed as U/mL.

Determination of reducing sugars

Released reducing sugars were measured by the 3,5-dinitrosalicylic acid method **(Miller, 1959)**.

Statistical analysis

All samples were analyzed in triplicate. The results were processed using the statistical program of Microsoft Excel 2010.

RESULTS AND DISCUSSION

Biomass sources (wheat straw, pine and poplar wood) were biotreated by *C. subvermispora* during 22 days of cultivation. Materials were selected for their different content of lignocellulosic components, because composition of lignocellulose can affect production of selected lignocellulolytic enzymes **(Galhaup *et al.*, 2002, Ferraz *et al.*, 2003; Souza – Cruz *et al.*, 2004)**. In our work **(Chmelová *et al.*, 2011)**, we found that organic sources of nitrogen (peptone, albumin) in cultivation medium used for enzyme production by *C. subvermispora* were the better than anorganic salts. Therefore, peptone was used as nitrogen source in all cultivation media.

Production of lignocellulolytic enzymes

C. subvermispora produced high levels of hydrolytic enzymatic activities during the entire biodegradation process (Fig 1 - 2). The highest cellulase production was reached on wheat straw (agricultural waste) and the lowest on pine wood (softwood). Cellulase activity increased during the initial cultivation period, reaching a peak value of 4.38 U/mL on the 8^{th} day of wheat straw pretreatment (Fig 1).

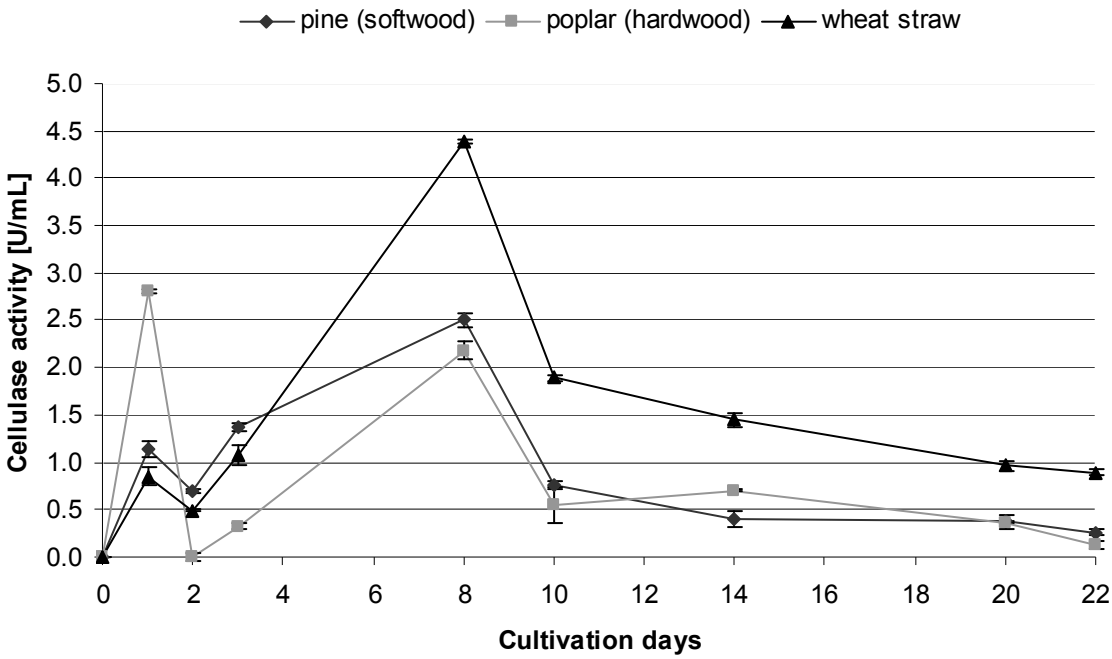

Figure 1 Time course for cellulase production by *C. subvermispora* on three different substrates during 22 days at 30 °C

Differences of cellulase activities reached in cultivation media containing various plant materials can be caused by their different composition. The highest cellulase activity was detected in medium with wheat straw, lower in medium with pine wood (softwood) and lowest cellulase activity was detected in medium with poplar wood (hardwood). The content of cellulose, hemicelluloses and lignin in used plant biomass **(Chmelová and Ondrejovič, 2010, unpublished data)** was in good accord with the results reported by **Demirbas (2005)**. Although cellulose content is higher in softwood and hardwood, these materials content also higher concentration of lignin. In the lignocellulose, lignin creates the main barrier to enzyme degradation of this material (**Sánchez, 2009**). Therefore, the lignin content determines both utilization of selected lignocellulose and enzyme production for degrading of selected component (cellulose, hemicelluloses, lignin). **Machuca and Ferraz (2001)** found out that degradation of cellulose and hemicelluloses is closely dependent on the extent lignin removal. Because white rot fungus *C. subvermispora* in the early days of lignocellulosic biodegradation did not produce laccases which degrade lignin, production of hydrolytic enzymes significantly decreased (Fig 1 – 2). Similarly results were observed in work **Tanaka *et al.* (2009).**

Table 2 Content of cellulose, hemicellulose and lignin in selected lignocellulosic materials **(Demirbas, 2005)**

Lignocellulosic material	Cellulose content [%]	Hemicellulose content [%]	Lignin content [%]
Straw (wheat)	32 - 39	29 - 38	16 – 17
Softwood (pine)	40 - 48	28 - 42	20 – 25
Hardwood (poplar)	41 - 47	22 - 32	25 - 33

The results of measured xylanase activities confirmed the above-mentioned hypothesis. Therefore, the highest xylanolytic activity was measured in cultivation medium with wheat straw, lower in medium with pine wood and lowest in medium with poplar wood. Increasing of xylanase activity correlates with concentration of hemicelluloses in selected plant material. This can be seen in Fig 2.

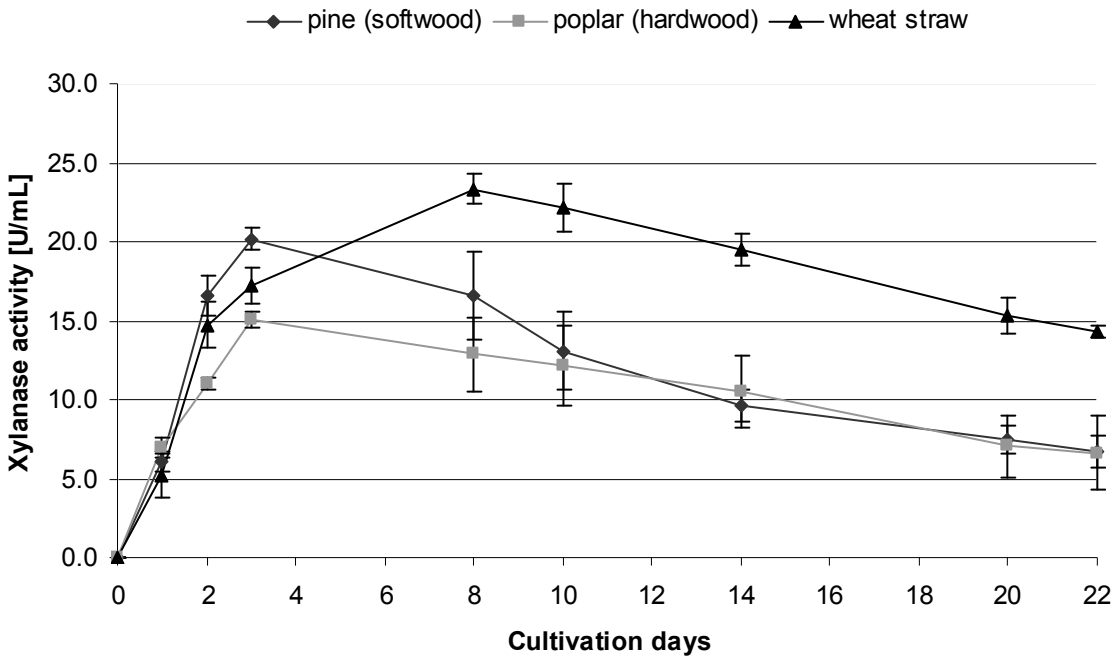

Figure 2 Time course for xylanase production by *C. subvermispora* on three different substrates during 22 days at 30 °C

On the 3rd day the xylanase activity decreased in medium with pine wood, wheat straw and poplar wood, respectively. Thereafter, the xylanase activity decrease in medium with pine and poplar wood and increase in medium with wheat straw that can be caused by faster degradation of straw lignocellulose and releasing of hemicelluloses from matrix. In the comparison of xylanase and cellulase is evident that production of xylanases was five times

higher than cellulase production. This result is achieved in works by other authors **(Ferraz *et al.*, 2003; Souza-Cruz *et al.*, 2004; Heidorne *et al*, 2006)**.

White rot fungus *C. subvermispora* by decreasing production of cellulases and xylanases begins to produce extracellular laccases into the cultivation medium and maximum of laccase activity correlates with lignin content in selected plant materials (Table 2). From our previously results **(Chmelová *at al.*, 2011)**, this increasing of laccase production is caused by exhausting of utilizable carbon sources. Laccase activity peaked after 16 days and then gradually decreasing (Fig 3). Decreasing of laccase activity may be caused by toxicity of lignin degradation products because the phenolic compounds located in polymer structure of lignin probably decrease both the *C. subvermispora* biomass grown and the production of laccases into cultivation medium **(Chmelová *at al.*, 2011)**. Similar results were described in work **Rüttimann-Johnson *et al.* (1992),** addition of veratryl alcohol in the cultivation medium of *C. subvermispora* decreased production of ligninolytic enzymes and decomposition of lignin were markedly decreased.

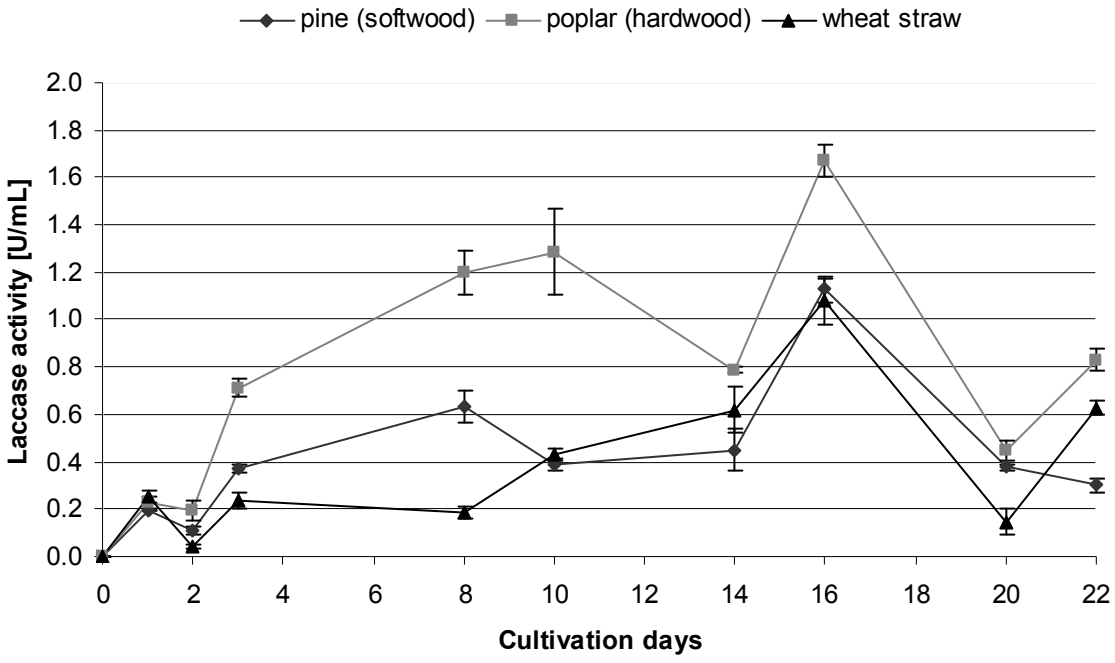

Figure 3 Time course for laccase production by *C. subvermispora* on three different substrates during 22 days at 30 °C

In the literature, expect lignin content, composition of lignin polymer structure affects of production ligninolytic enzymes. Poplar, the best substrate for laccase production, belongs to hardwood materials which contain notably syringyl and guaiacyl monomers of lignin,

whereas softwood, for example pine wood, contains primarily guaiacyl monomers of lignin **(Eaton and Hale, 1993)**. Guaiacyl structures are degraded more easily than syringyl structures of lignin **(Kirk *et al.*, 1975)**.

CONCLUSION

We found that composition of the lignocellulosic material (wheat straw, pine and poplar wood) affects the production of lignocellulolytic enzymes by white rot fungus *Ceriporiopsis subvermispora*. Significant effect to lignocellulolytic enzyme production has content and composition of lignin because this polymer allows subsequent degradation other polymers in the lignocellulose. During cultivation of *C. subvermispora* on selected lignocellulosic materials, degradation of particular polymers was observed. Lignocellulolytic enzymes achieved maximum of their activity in the order xylanase, cellulases and laccases. This finding indicates succession of steps which white rot fungus *C. subvermispora* degrades colonized lignocellulose in nature.

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