Arch. Biol. Sci., Belgrade, 58 (3), 179-182, 2006.

EFFECT OF MEDIUM pH AND CULTIVATION PERIOD ON MYCELIAL BIOMASS, POLYSACCHARIDE, AND LIGNINOLYTIC ENZYME PRODUCTION BY *GANODERMA LUCIDUM* FROM MONTENEGRO

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Abstract - The effect of initial medium pH on biomass, extracellular and intracellular polysaccharide, and ligninolytic enzyme production by *Ganoderma lucidum* was investigated at different pH values after 7 and 14 days of cultivation. Maximal production of biomass was recorded at pH 4.5 and 5.0; maximal production of extracellular polysaccharides at pH 7.0 and 3.0; and maximal production of intracellular polysaccharides at pH 7.0 and 5.5. Ligninolytic enzymes were not produced at any pH of the medium. Maximal biomass production was obtained on the 11th day of cultivation; maximal extracellular polysaccharide production on the 7th day; and maximal intracellular polysaccharide production on the 6th and 10th day of cultivation.

Key words: *Ganoderma lucidum*, polysaccharides, ligninolytic enzymes, medium pH, mycelial biomass, Montenegro

UDC 57.04:582.28
577.15:582.28

INTRODUCTION

Ganoderma lucidum has been used in medicine of the Far East for more than 2000 years (S t a m e t s, 1993) for treatment of various human diseases, such as chronic hepatitis, hypertension, hypercholesterolemia, hyperglycemia, coronary heart disease, bronchial asthma, duodenal ulcer, etc. (H o b b s, 1995).

The fruiting bodies and mycelium of *G. lucidum* contain immunomodulating polysaccharides, some of which inhibit the growth of several cancer cells (S o n e *et al*., 1985). In chemical structure the polysaccharides are 1,3-b-D-glucans that contain a large number of D-glucose molecules linked by glycoside bonds and which are highly branched (W a g n e r *et al*., 2004). Z h a n g *et al.* (2001) found a positive correlation between the degree of polysaccharide branching and their immunomodulating effects.

The role of polysaccharides in mushroom metabolism has not yet been fully recognized. According to K r c m a r *et al.* (1999), extracellular polysaccharides play an important role in the process of lignin degradation as an indirect source of hydrogen peroxide and in maintaining optimal pH for ligninolytic enzyme production. They also function as a supporting network on which some of the excreted enzymes adsorb.

The aims of this research were to determine the optimal initial medium pH for production of biomass, polysaccharides, and ligninolytic enzymes, as well as to study the dynamics of their synthesis.

MATERIAL AND METHODS

Organism and inoculum preparation

Ganoderma lucidum BFB 32 from a conifer forest near Rožaje (Montenegro) was used. It is currently maintained on malt agar medium at 4ºC in the culture collection of the Institute of Botany, Faculty of Biology (BFB), Serbia.

For preparation of the inoculum, 25e mycelial agar disks (0.5 cm) were used for inoculation of 100 ml of synthetic medium (K o *et al.*, 2001) which was incubated for 14 days at the room temperature (22 ± 2ºC) on a rotary shaker at 160 rpm. The obtained biomass was washed three times with sterile distilled water and homogenized

with 100 ml of sterile distilled water in a laboratory blender.

Influence of different medium pH values on mycelial biomass, polysaccharide, and ligninolytic enzyme production

The cultivation was performed in 250 ml flasks containing 50 ml of synthetic medium with medium pH values of 2.0, 3.0, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, and 7.0 at room temperature on a rotary shaker at 160 rpm. Three repetitions were performed for each pH value.

The homogenized inoculum (5 ml per flask) was used for inoculation. After 7 to 14 days of cultivation, the mycelial biomass was separated by centrifugation (4°C, 3000 rpm, 30 min), dried, and weighed. The obtained supernatant was dialyzed and used to determine extracellular polysaccharide (EPS) production. Crude EPS were precipitated by adding four volumes of 95% ethanol at 4°C during the night, then separated by centrifugation (4°C, 3000 rpm, 10 min). The produced EPS were dried at 50°C to constant weight and presented as $mg \cdot ml^{-1}$ of supernatant.

Produced intracellular polysaccharides (IPS) were extracted from dried, frozen, and macerated mycelium by cooking in 10 ml of distilled water at 100°C for one hour. After cooling, the supernatant was separated by centrifugation (4°C, 3000 rpm, 30 min) and dialyzed. Precipitation of IPS was achived by adding four volumes of 95% ethanol. The further procedure was the same as for EPS. The amount of produced IPS is presented as $mg \cdot g^{-1}$ of dried mycelial weight (d.w.).

The investigated ligninolytic enzymes were laccase (Lac), Mn-dependent peroxidase (MnP), and versatile peroxidase (VP). Their activities were determined spectrophotometrically. Laccase activity was assayed using syringaldazine ($\varepsilon_{525} = 65000\ M^{-1} \cdot cm^{-1}$) (S t a j i ć *et al.*, 2004), while peroxidase activity was determined with 3 mM phenol red ($\varepsilon_{610} = 22000\ M^{-1} \cdot cm^{-1}$) (S t a j i ć *et al.*, 2006).

Enzymatic activity of 1 U was defined as the amount of enzyme that transforms 1 µmol of substrate/min. A UV-160A Spectrophotometer (Shimaden) was used for these assays.

Influence of cultivation period on mycelial biomass and polysaccharide production

The cultivation was performed in 250-ml flasks with 50 ml of synthetic medium, pH 4.5 (the optimal pH for mycelial biomass production), which were inoculated with 5 ml of homogenized inoculum and incubated at room temperature on a rotary shaker at 160 rpm. The measurements of mycelial biomass, EPS, and IPS production were performed daily from the 4th to 13th day of cultivation. Three repetitions per day of cultivation were carried out.

RESULTS AND DISCUSSION

Influence of different medium pH values on mycelial biomass, polysaccharide, and ligninolytic enzyme production

Mycelial biomass production at low initial medium pH values (2.0 and 3.0) was not significant (1.40±0.20 and 0.60±0.01 $g \cdot l^{-1}$ of the medium, respectively). With increase of pH, it rose to a maximum at pH 4.5 (8.80±2.40 $g \cdot l^{-1}$ of the medium) on the 7th day of cultivation (Fig. 1). After the obtained peak, further increase of pH resulted in an initial sharp and later slight decline of biomass production, which then remained at approximately the same level (2.40 $g \cdot l^{-1}$ of the medium). At 14 days of cultivation, a peak of biomass production (20.60±0.60 $g \cdot l^{-1}$ of the medium) was obtained at pH 5.0, after which it declined with further increase of pH (Fig. 1). These results are not in accordance with the results of F a n g and Z h o n g (2002) and H s i e h *et al.* (2005), who obtained maximal mycelial biomass production at initial medium pH values of 6.0 and 6.5.

Production of EPS varied during cultivation. It was very low at low initial pH, while maximal production was obtained at initial pH of 7.0 on the 7th day of cultivation (2.48±0.33 $mg \cdot ml^{-1}$ of supernatant) and at initial pH of 3.0 on the 14th day of cultivation (5.20±0.85 $mg \cdot ml^{-1}$ of supernatant). At higher initial pH values EPS synthesis was greatly reduced (Fig. 2).

Y a n g and L i a u (1998) found that the optimal initial

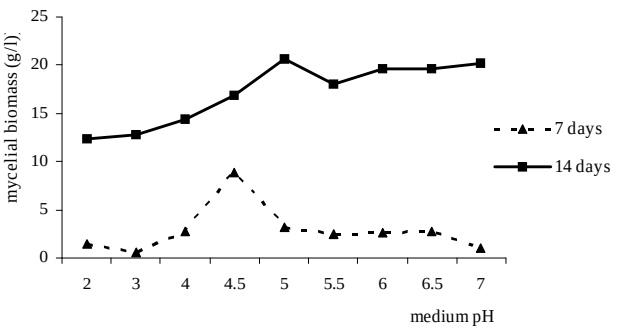

Fig. 1. Influence of initial medium pH on mycelial biomass production.

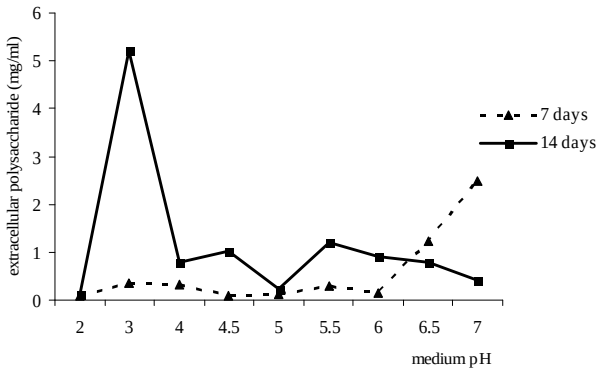

Fig. 2. Influence of initial medium pH on extracellular polysaccharide production.

values of pH of the medium for EPS synthesis were between 4.0 and 4.5, both after 7 and after 14 days of cultivation. However, F a n g and Z h o n g (2002) obtained the highest level of EPS production at initial pH of 5.5 after 8 days and at initial pH of 3.5 after 14 days of cultivation.

The dependence of IPS production on initial medium pH was different from that obtained for EPS production. After 7 days of cultivation, maximal IPS synthesis was obtained at initial pH of 7.0 (69.44±11.34 mg·g^{-1} d.w.); after 14 days it was obtained at initial pH of 5.5 (69.93±1.62 mg·g^{-1} d.w.) (Fig. 3). F a n g and Z h o n g (2002) also found that relatively high initial pH values (5.5-7.0) were optimal for IPS production.

Production of ligninolytic enzymes did not occurr at any of the analyzed values of initial medium pH. However, K o *et al.* (2001) obtained three Lac isozymes using the same synthetic medium over a wide pH range. To judge from the data of S t a j i ć *et al.* (2004), who showed that production of these enzymes depends on the mushroom species and strain, cultivation conditions

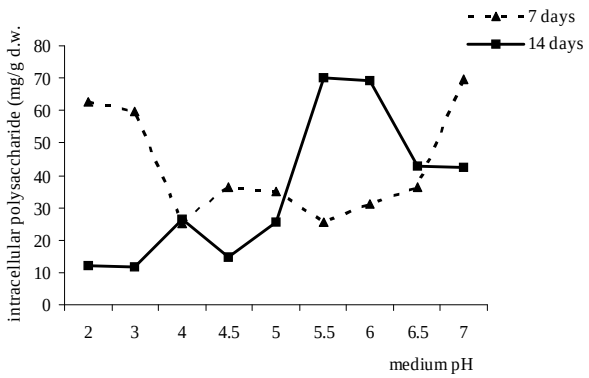

Fig. 3. Influence of initial medium pH on intracellular polysaccharide production.

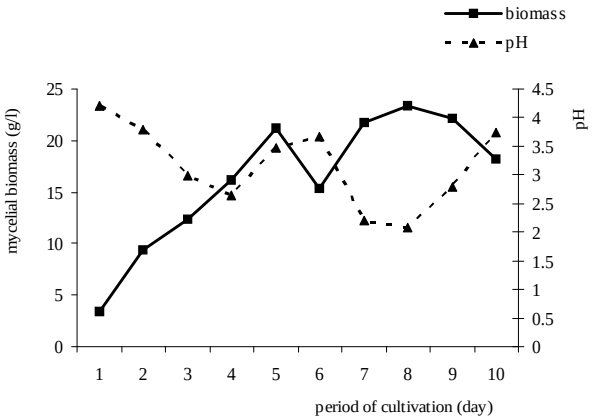

Fig. 4. Influence of cultivation period on mycelial biomass production and changes of medium pH.

(submerged or solid-state), and the carbon and nitrogen sources in the medium, it can be concluded that composition of the medium we used was not suitable for enzyme production by the analyzed *G. lucidum* strain.

Influence of cultivation period on mycelial biomass and polysaccharide production

Biomass production accelerated from the 4th (3.40±0.26 g·l^{-1} of the medium) to the 11th day of cultivation, when it reached its maximum (23.40±0.60 g·l^{-1} of the medium) (Fig. 4). These results showed that the first significant value of produced mycelial biomass was obtained at the 8th day of cultivation (21.20±1.40 g·l^{-1} of the medium), which is in accordance with the results of Fang and Zhong (2002). Changes of medium pH were also measured and found to decline in correlation with increase of biomass production from the 4th to the 7th day of cultivation (4.20; 3.79; 2.98; 2.64), as well as from the 9th to the 11th day (3.67; 2.20; 2.07) (Fig. 4).

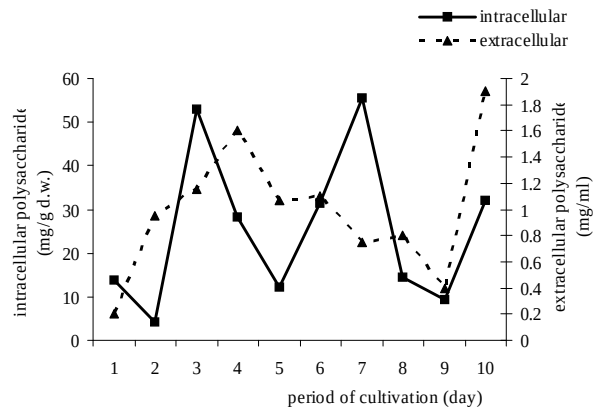

Fig. 5. Influence of cultivation period on extra- and intracellular polysaccharide production.

Production of EPS increased from the 4th to the 7th day of cultivation, when maximal production was reached (1.60±0.08 $mg \cdot ml^{-1}$ of supernatant); after that, it gradually decreased, which is in accordance with the results of Fang and Zhong (2002). On the other hand, IPS production showed two peaks, at the 6th and 10th days of cultivation (52.84±7.41 and 55.46±19.32 $mg \cdot g^{-1}$d.w., respectively) (Fig. 5).

The results suggest that the cultivation conditions (initial pH of the medium and period of cultivation) are significant for accumulation of mycelial biomass, as well as for EPS and IPS production.

Acknowledgement - This work was supported by the Serbian Ministry of Science and Environment Protection (Grant 143041).

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ЕФЕКАТ pH ПОДЛОГЕ И ДУЖИНЕ КУЛТИВАЦИЈЕ НА ПРОДУКЦИЈУ МИЦЕЛИЈЕ, ПОЛИСАХАРИДА И ЛИГНИНОЛИТИЧКИХ ЕНЗИМА КОД *GANODERMA LUCIDUM* ИЗ ЦРНЕ ГОРЕ

ЈЕЛЕНА ВУКОЈЕВИЋ, МИРЈАНА СТАЈИЋ, СОЊА ДУЛЕТИЋ-ЛАУШЕВИЋ и ЈАСМИНА СИМОНИЋ

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Циљ ових истраживања био је проучавање ефекта различитих почетних pH подлоге на продукцију мицелије, екстра- и интрацелуларних полисахарида, као и лигнинолитичких ензима код *Ganoderma lucidum* после седам и 14 дана култивације. Максимална продукција биомасе мицелије је била забележена на pH 4,5 (после седам дана култивације), и на pH 5,0 (после 14 дана култивације). Максимална продукција екстрацелуларних полисахарида је добијена на pH 7,0 и pH 3,0, док је пик синтезе интрацелуларних полисахарида уочен при pH 7,0 седмог дана култивације, односно при pH 5,5 четрнаестог дана култивације. Продукција лигнинолитичких ензима се није десила ни при једној анализираној pH вредности подлоге. Утицај дужине култивације је био проучаван при оптималној pH подлоге за продукцију мицелије и мерења су била урађена почев од четвртог до 13 дана култивације. Максимална продукција биомасе је добијена 11 дана, екстрацелуларних полисахарида седмог дана, а интрацелуларних полисахарида шестог и 10 дана култивације.