Influence of Trace Elements on Ligninolytic Enzyme Activity of *Pleurotus ostreatus* and *P. pulmonarius*

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Trace elements, at tolerable concentrations, are either part of the active site or act as an activity modulator of ligninolytic enzymes of white-rot mushrooms. They are usually in plant raw materials in a non-toxic amount or non-available and extractable forms. This study evaluated the effects of Fe, Zn, and Se on the activity of laccase and Mn-oxidizing peroxidases of *P. ostreatus* and *P. pulmonarius* during solid-state fermentation of grapevine sawdust. The studied species showed different levels of tolerance to the trace elements. A stimulatory effect of the microelements on laccase activity was demonstrated in *P. ostreatus*, while Fe and Zn were strong inhibitors of the activity of *P. pulmonarius*, which was contrary to Se, independent of the cultivation period. With the exception of SeO_2, activity of Mn-oxidizing peroxidases in *P. ostreatus* was suppressed in various levels by the elements. However, in *P. pulmonarius*, activity against phenol red oxidation in the presence of external Mn^{2+} was stimulated by the elements on day 7, while on day 10, activity inhibition by Fe and Zn and stimulation by Se was noted. The effect on activity against phenol red oxidation in the absence of external Mn^{2+} was the opposite.

Keywords: Grapevine sawdust; Iron; Laccase; Mn-oxidizing peroxidases; Pleurotus ostreatus; P. pulmonarius; Selenium; Solid state fermentation; Zink

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INTRODUCTION

Growth of world population and industrialization have led to rise in demand for food, fibers, and fuels, but have also resulted in an enormous annual production of plant biomass. Nowadays, 97% of plant residues remain unutilized (Zechendorf 1999) and up to 80% of the primary energy is obtained from fossil fuels (Nigam and Singh 2011). Recently, special attention is given to plant biomass transformation in fibers and biofuels, which are in accordance with current trends of forest preservation, finding an environmentally friendly energy source, and raw materials conversion. However, apart from the final products, the efficiency of the processes depends on the potential of the organisms introduced in waste degradation and cultivation conditions. Mushrooms, especially white-rot species, are the main degraders. Species of the genus *Pleurotus* possess a potent ligninolytic enzyme system, which enables successful degradation of lignin and various aromatic compounds, and allows for participation in different biotechnological processes. Grapevine sawdust is a very common agricultural waste in

some regions and is a prospective substrate for the bioconversion into fungal biomass and lignocellulolytic enzymes due to chemical composition (Kilby 1999; Stajić *et al*. 2006).

Some trace elements are essential for the fungal metabolism due to their location in the enzyme active site or their modulation of enzyme activities. However, in greater concentrations they could be toxic (Baldrian and Gabriel 2002; Baldrian 2003). Additionally, trace elements such as Fe, Zn, and Se could act through metal-responsive promoter interaction, to have posttranscriptional regulation and to cause oxidative stress (Thiele 1992; Faraco *et al*. 2003; Catal *et al*. 2008). Microelements in the plant raw materials are either present in a non-toxic amount, or they are not available, or they are in extractable forms. Because of this, the residues are a prospective substrate for fungal growth (Baldrian and Gabriel 2002; Baldrian *et al.* 2005). Compared with submerged fermentation, solid-state fermentation provides certain advantages of fungal enzyme production (Viniegra-González *et al.* 2003) with the aspect of application in bioprocesses such as biobleaching, biopulping, bioremediation, *etc*. (Pandey 2003). Since the effect of different nitrogen sources on ligninolytic enzyme production by *Pleurotus* species have been studied during solid-state fermentation of grapevine sawdust, showing a promising potential in biotechnological applications (Stajić *et al*. 2006), the question remains whether medium enrichment with trace elements could improve the enzyme activity.

Previous data influenced formulation of the aim of the study and the analysis of the effects of form and concentration of the selected trace elements on ligninolytic enzyme activity of *P. ostreatus* and *P. pulmonarius.* Attention was placed on the fermentation of grapevine sawdust.

EXPERIMENTAL

Materials

The cultures of *Pleurotus ostreatus* HAI 494, cultivated strain from Hawaii (USA) and *P. pulmonarius* HAI 572, collected in Ukraine, were obtained from the culture collection of the Institute of Evolution, University of Haifa, Israel (HAI), and preserved in the culture collection at the Institute of Botany, Faculty of Biology, University of Belgrade.

Methods

Growth conditions

The inoculum preparation involved the following steps: *(i)* inoculation of 100 mL of synthetic medium (glucose, 10.0 g/L; NH_4NO_3, 2.0 g/L; K_2HPO_4, 1.0 g/L; NaH_2PO_4 x H_2O, 0.4 g/L; $MgSO_4$ x $7H_2O$, 0.5 g/L; yeast extract, 2.0 g/L; pH 6.5) with 25 mycelial discs (Ø 0.5 cm, from 7 day-old culture from malt agar); *(ii)* incubation at room temperature (22 ± 2 ºC), on a rotary shaker (160 rpm), for 7 days; *(iii)* washing of obtained biomass (3 times) by sterile distilled water; and *(iv)* biomass homogenization with 100 mL of sterile distilled water in laboratory blender.

Solid-state cultivation was carried out at 25 ºC in 100 mL-flasks containing 4 g of grapevine sawdust, and 12 mL of the modified synthetic medium (without glucose, with nitrogen in a form of peptone and amount of 0.5% for *P. ostreatus* HAI 494, and in a

form of NH_4NO_3 and concentration of 30 mM for *P. pulmonarius* HAI 572). The used nitrogen forms and concentrations were already demonstrated as optimal for activity of selected enzymes under mentioned conditions (Stajić *et al.* 2006a). Testing trace elements were Fe, Zn, and Se in the forms of $FeSO_4$ x 7 H_2O, $ZnSO_4$ x 7 H_2O, Na_2SeO_3, Na_2SeO_4, and SeO_2, and concentration of 1 mM. The medium without microelements was used as the control.

Inoculation was done by usage of 3 mL of inoculums homogenate per flask. Samples were harvested after 7 and 10 days of cultivation, and the ligninolytic enzymes were extracted by stirring of samples with 50 mL distilled water on magnetic stirrer for 10 min at 4 ºC. The obtained extracts were separated by centrifugation (4 ºC, 5000 rpm, 15 min) and supernatants were used for measurements of activities of laccase (EC 1.10.3.2), Mn-dependent peroxidase (MnP, EC 1.11.1.13), and versatile peroxidase (VP, EC 1.11.1.16). Three replicate flasks for each sampling occasion were analyzed.

Enzyme activity assays

Activity of the ligninolytic enzymes was determined spectrophotometrically. Laccase activity was assayed using syringaldazine (4-hydroxy-3,5-dimethoxy-benzaldehyde azine) and by measuring the increase in absorbance at 525 nm (ε_{525} = 65000 $M^{-1}cm^{-1}$) for 60 seconds (Stajić *et al.* 2004). The mixture contained 0.1 M acetic buffer (pH 5.0), 1 mM syringaldazine (dissolved in 96% ethanol), and sample (V_{tot} = 1 mL).

Mn-oxidizing peroxidases activities were defined with 3 mM phenol red (ε_{610} = 22000 $M^{-1}cm^{-1}$) in a 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 VP, respectively), and the reaction was stopped by 2 M NaOH (Stajić *et al.* 2006, 2010).

Enzymatic activity of 1 U is defined as the amount of enzyme that transforms 1 μM of substrate per min. Three measurements of the activity per each flask were done.

Statistical analysis

The results were expressed as the mean ± standard error of data obtained from triplicate experiments. One-way analysis of variance (ANOVA) was used to test the significance of differences among the species and trace elements using STATISTICA software, version 5.0 (StatSoft, Inc). P-values less than 0.01 were considered statistically significant.

RESULTS

Effect of Trace Elements on Laccase Activity

Laccase activity was detected in crude extracts of 7- and 10-day-old *P. ostreatus* HAI 494 and *P. pulmonarius* HAI 572 cultures, which were grown in the media unenriched and enriched with microelements (Fig. 1). After 7 days of cultivation, in comparison with the control, the presence of Fe and Zn caused a slight increase of

laccase activity in *P. ostreatus* HAI 494 (16.5% and 13.1%, respectively), and a slight decrease in *P. pulmonarius* HAI 572 (3.6% and 4.3%, respectively) (Fig. 1). However, on day 10 of cultivation, the level of laccase activity was lower compared with the control, except in *P. ostreatus* HAI 494 in Zn-enriched medium (252.7 U/L in the control and 632.0 U/L in the presence of Zn, which is 2.5-fold higher). Cultivation period caused a significant reduction of the activity in the control and in the presence of Fe and Zn ($P<0.01$), except in *P. ostreatus* HAI 494 in Zn-enriched medium. The most significant activity decrease was noted in *P. pulmonarius* HAI 572 in the presence of Fe and was approximately 3-fold lower on day 10 than on day 7 (from 289.9 U/L to 91.1 U/L). Generally, *P. ostreatus* HAI 494 was better laccase producer than *P. pulmonarius* HAI 572 as in the control as in the medium enriched with Fe or Zn (Fig. 1).

The tested Se forms and cultivation period influenced laccase activity ($P<0.01$) in various ways. Compared to the control, after 7 days, activity increased in both species in the media enriched with all tested Se forms, except in *P. ostreatus* HAI 494 in the presence of Na_2SeO_3, where a decrease of 7.4% was noted (384.3 U/L). On day 10, various levels of the activity increase were obtained in comparison with the control. Depending on the cultivation period, Na_2SeO_3 and SeO_2 showed a slight inhibitory effect on the activity in *P. ostreatus* HAI 494 and a stimulatory effect on *P. pulmonarius* HAI 572. However, influence of Na_2SeO_4 was stimulatory in both species; in *P. ostreatus* HAI 494, an increase of 15.4% was detected on day 7 (478.9 U/L), and an increase of almost 2-fold was detected on day 10 (746.1 U/L). In *P. pulmonarius* HAI 572, an increase of 15.6% (347.5 U/L) on day 7, and an increase of 29.4% (175.1 U/L) on day 10, were detected (Fig. 1).

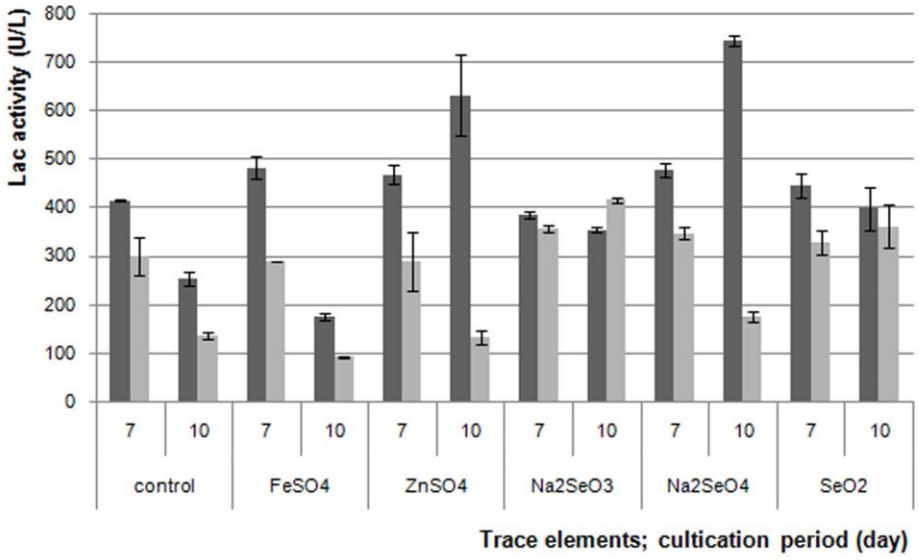

Fig. 1. Influence of microelements and cultivation period on laccase activity in *Pleurotus ostreatus* (■) and *P. pulmonarius* (□) during solid state fermentation of grapevine sawdust. The values in the figure correspond to mean values ± S.E of three replicates.

Effect of Trace Elements on the Activity of Mn-oxidizing Peroxidases

Activities of Mn-oxidizing peroxidases were also noted in the crude mixture after grapevine sawdust fermentation by *P. ostreatus* HAI 494 and *P. pulmonarius* HAI 572, in the absence, as well as in the presence of tested trace elements (Fig. 2).

The level of phenol red oxidation in the presence of external Mn^{2+}, after 7 days of *P. ostreatus* HAI 494 cultivation, decreased in the presence of Fe and Zn by 18.2% and 41.6%, respectively, in comparison with the control (7.7 U/L). This oxidation level in *P. pulmonarius* HAI 572, in the control medium, was approximately 2.5-fold lower than in *P. ostreatus* HAI 494 (2.9 U/L and 7.7 U/L, respectively), while in the medium enriched with Fe and Zn, an insignificant increase of activity was noted (from 2.9 U/L to 3.7 U/L and 3.6 U/L, respectively). On day 10 of cultivation, activities against phenol red in the presence of external Mn^{2+} in the control were similar in both species (7.7 U/L, in *P. ostreatus* HAI 494, and 7.2 U/L, in *P. pulmonarius* HAI 572). However, activity of *P. ostreatus* HAI 494 exhibited the same value as on day 7, while in *P. pulmonarius* HAI 572 it was higher twice. In comparison with the control, activities decreased in the presence of Fe and Zn after 10 days of cultivation (Fig. 2A). A slight stimulatory effect of cultivation period was noted in *P. ostreatus* HAI 494, and it was more significant in *P. pulmonarius* HAI 572, especially in the control. Generally, *P. ostreatus* HAI 494 was a better oxidizer of phenol red in the presence of the external Mn^{2+}, in the control, and in the presence of mentioned trace elements ($P<0.01$) (Fig. 2A).

After 7 days of cultivation, in comparison with the control, the tested Se forms caused a reduction of phenol red oxidation level in the presence of the external Mn^{2+} in *P. ostreatus* HAI 494 (from 7.7 U/L to 4.8 U/L in the SeO_2-amended medium, which is 37.7% lower), while in *P. pulmonarius* HAI 572, the decrease level was lower (5.2 U/L in the SeO_2-enriched medium, which is 27.8% lower). On day 10, in *P. pulmonarius* HAI 572, presence of Se in the medium caused a slight increase of activity against phenol red in the presence of the external Mn^{2+} compared to the control (values ranged between 7.3 U/L and 8.9 U/L, which is 1.4% and 23.6% higher, respectively), while in *P. ostreatus* HAI 494, activity decreased in Na_2SeO_4-enriched medium (6.6 U/L or 14.3% lower) and increased in the SeO_2-amended medium (10.3 U/L or 33.8% higher). *P. ostreatus* HAI 494 was a better producer of this enzyme after 7 days of cultivation, and *P. pulmonarius* HAI 572 after 10 days of cultivation, except in the case of SeO_2-enriched medium. The form of Se did not significantly influence activity of the enzyme (Fig. 2A).

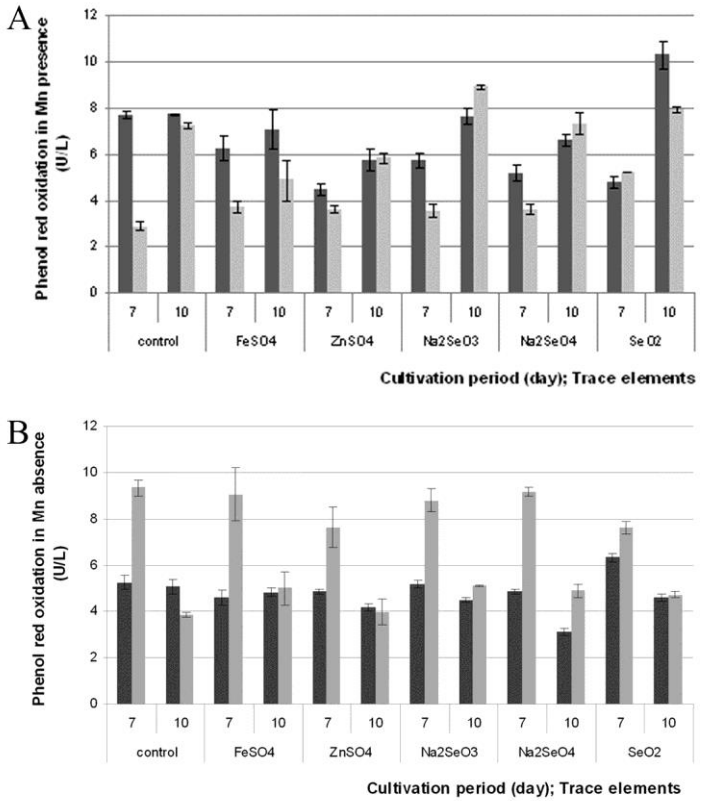

Fig. 2. Influence of microelements and cultivation period on Mn-oxidizing peroxidase activity in *Pleurotus ostreatus* (■) and *P. pulmonarius* (□) during solid state fermentation of grapevine sawdust. **A.** Mn-dependent peroxidase, **B.** Versatile peroxidase. The values in the figure correspond to mean values ± S.E of three replicates.

On day 7 of cultivation, in both species, activity against phenol red in the absence of the external Mn^{2+} was lower in the presence of Fe or Zn compared with the control. Zn was a stronger inhibitor of the activity, especially in *P. pulmonarius* HAI 572 (9.3 U/L in the control and 7.6 U/L in Zn-enriched medium, which is 18.3% lower). *P. pulmonarius* HAI 572 was a significantly better enzyme producer than *P. ostreatus* HAI 494, in the control and in Fe- and in Zn-enriched medium ($P<0.01$) (Fig. 2B). Cultivation period did not affect the level of phenol red oxidation in the absence of the external Mn^{2+} in *P. ostreatus* HAI 494, while in *P. pulmonarius* HAI 572, an activity decrease of 50% was noted both in the control and in the medium with Fe or Zn (Fig 2B).

In comparison to the control, the tested Se forms did not significantly influence the activity against phenol red oxidation in the absence of the external Mn^{2+}, after 7 days of cultivation, except in the presence of SeO_2, which caused an increase of 18.9% in *P. ostreatus* HAI 494 (5.3 U/L and 6.3 U/L, respectively) and a decrease of 18.3% in *P. pulmonarius* HAI 572 (9.3 U/L and 7.6 U/L, respectively). Cultivation period variously affected production of the enzyme. In *P. ostreatus* HAI 494, in the control, differences were at the statistical error level (5.3 U/L on day 7 and 5.1 U/L on day 10), while the activity decrease was noted in Se-enriched media. However, an inhibitory effect was noted in *P. pulmonarius* HAI 572 especially in the control (approximately 41%) (Fig. 2B). Although *P. pulmonarius* HAI 572 was a better producer of the enzyme on day 7 of

grapevine sawdust fermentation, the presence of the tested trace elements did not significantly affect production of the enzyme ($P>0.01$).

DISCUSSION

A number of environmental factors could significantly affect production of ligninolytic enzymes in white-rot mushrooms. Among those external factors, trace elements are recognized as agents that can interact with the enzymes or participate in gene regulation processes, mainly on a transcriptional level (Baldrian 2003; Piscitelli *et al*. 2011). Generally, these elements in low concentrations are necessary for synthesis and function of the ligninolytic enzymes, while their higher amounts present potent inhibitors of enzymatic reactions (Vallee and Ulmer 1972). Despite advantages of the submerged cultivation, homogenous distribution of solvents, and easier trace element intake into fungal cells, conditions of plant residues solid-state fermentation are similar to nature conditions.

Effect of Trace Elements on Laccase Activity

A stimulatory effect of Zn on laccase activity, noted in *P. ostreatus* HAI 494, was also demonstrated in *Trametes versicolor* and *T. hirsuta* (Collins and Dobson 1997; Keum and Li 2004), while in *P. pulmonarius* HAI 572 and other tested strains, as well as in *T. pubescens* this element reduced the activity (Galhaup and Haltrich 2001; Tychanowicz *et al.* 2006), which could be explained by species specificity. Laccase induction could be the result of limited selectivity of the metal-responsive promoter in the laccase gene in *P. ostreatus*, in which transcription can be controlled by several metal ions, including Zn (Faraco *et al.* 2003). These authors explained the inhibitory effect of Zn by reduction of its affinity to bind to metal-regulatory protein that functions as a transcription factor. Lakewise, Baldrian and Gabriel (2002) and Baldrian (2004) showed that the effect of this microelement on enzyme activity depends on cultivation type.
According to Das *et al.* (2001), Hatvani and Mécs (2003), and Rodríguez Couto *et al.* (2005), an inhibitory effect of Fe on laccase activity was expected. Both tested species were sensitive to this trace element, with a higher resistance level in *P. ostreatus*. The dual nature of the effect of Fe and Zn on laccase activity, induction or suppression, could be explained by a genetic basis of response to the presence of these metals.

Se in all tested forms was a stimulator of laccase activity. Muñoz *et al.* (2006) offers a possible explanation for *P. ostreatus*, where the stimulatory effect of lower Se concentrations on mycelial growth was demonstrated, contrary to higher concentration. Another interpretation of laccase induction could be the response to the stress caused by excessive amount of Se, namely laccase is involved in the synthesis of pigments which prevent the uptake of trace elements (Galhaup and Haltrich 2001).

Effect of Trace Elements on the Activity of Mn-oxidizing Peroxidises

Some trace elements are also involved in the transcriptional regulation of genes for Mn-oxidizing peroxidases through transcriptional factors that specifically interact with DNA sequences in the upstream region of the target genes (Thiele 1992; Manubens

et al. 2003). Since these factors are involved in Fe free transport, its uptake and utilization in fungal cells is highly genetically controlled. In such a way, high Fe concentrations, which represent stress conditions, affect ligninolytic enzyme production. Zn also regulates peroxidase genes on both a transcriptional and posttranslational level through various regulatory mechanisms (Thiele 1992). However, enhancement of MnP gene transcription does not necessarily lead to exceeded MnP activity, confirming the lack of a relationship between mRNA levels and extracellular MnP activity (Manubens *et al.* 2003).

According to the obtained results, a concentration of 1 mM of Fe and Zn in the medium presents a moderately higher concentration, causing a slightly negative effect on the activity against phenol red oxidation in the presence of the external Mn^{2+}, compared to the control. Concentration of these trace elements in the grapevine sawdust, used as a unique carbon source, is significant (Kilby 1999). However, it does not mean that the amount of microelements is extractable and available completely (Baldrian and Gabriel 2002; Baldrian *et al.* 2005). For instance, Baldrian *et al.* (2005) noted that only 44% Zn and 3% Fe of total amount in wheat straw were used by *P. ostreatus*.

An increase of the activity in *P. pulmonarius* HAI 572 in the presence of Se is in consonance with previously published results for *Bjerkendera adusta* (Catal *et al.* 2008) and *P. eryngii* (Stajić and Vukojević 2011). Contrary to *B. adusta*, in which enzyme activities were significantly inhibited at concentrations between 0.1 mM and 0.2 mM, *Pleurotus* species have shown higher tolerance and a significant level of Mn-oxidizing peroxidases activity at a concentration of 1 mM.

The possible explanation for the slightly inhibitory effect of Se on peroxidises in *P. ostreatus* HAI 494 could lie in oxidative stress, which is induced by higher concentrations (Stewart *et al.* 1999; Catal *et al.* 2008). The stimulatory effect of Se on activity against phenol red oxidation in the absence of the external Mn^{2+}, demonstrated in *P. eryngii* (Stajić and Vukojević 2011), was not proven in the studied species. One possible explanation is that the level of Mn-oxidizing peroxidases activity depends on Se influence on gene regulation. Thus, low intracellular Se concentrations do not induce any significant change in their activities *via* gene control (Muñoz *et al.* 2006; Catal *et al.* 2008).

The effect of various trace elements on ligninolytic enzyme activity and mechanisms on which they act during regulation of enzyme synthesis remain the subject of the current and future research. Addressing the questions regarding the significant influence of trace elements on ligninolytic enzymes during numerous industrial processes, such as decolorization and degradation of pollutants, and giving valuable answers on those questions presents a road map to improvement of industrial application of those enzymes.

CONCLUSIONS

1. The difference in tolerance of trace elements is species specific.
2. Fe, Zn, and Se stimulate laccase activity in *P. ostreatus* HAI 494.
3. Fe, Zn, and Se suppress activity against phenol red oxidation in the presence of the external Mn^{2+}, in *P. ostreatus* HAI 494, and SeO_2 is a stronger inhibitor on day 7 and stimulator on day 10 of cultivation. The effect of the trace elements is the same on the activity against phenol red oxidation in the absence of the external Mn^{2+}, with the exception of SeO_2, where it is opposite.
4. Studied trace elements stimulate activity against phenol red oxidation in the presence of the external Mn^{2+} in *P. pulmonarius* HAI 572 in early stage of cultivation, while later, the activity level is lower in the presence of Fe and Zn and higher with Se enhancement.

ACKNOWLEDGEMENT

This study was carried out with financial support of Ministry of Education, Science, and Technological Development of Republic of Serbia, project No. 173032.

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Article submitted: March 6, 2013; Peer review completed: April 9, 2013; Revised version received and accepted: April 29, 2013; Published: April 30, 2013.