WHEAT STRAW CONVERSION BY ENZYMATIC SYSTEM OF *GANODERMA LUCIDUM*

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The purpose of this study was to resolve the question of whether various nitrogen sources and concentrations affect characteristics of selected *G. lucidum* ligninolytic enzymes participating in wheat straw fermentation. This is the first study reporting the presence of versatile peroxidase activity in crude extract of *G. lucidum* culture, as well as isoforms profile of Mn-oxidizing peroxidases. NH_4NO_3 was the optimum nitrogen source for laccase and Mn-dependent peroxidase activity, while peptone was the optimum one for versatile peroxidase activity. Four bands with laccase activity were obtained by native PAGE and IEF separations from medium enriched with inorganic nitrogen source, and only two bands from medium containing organic source. Medium composition was not shown to affect isoenzyme patterns of Mn-oxidizing peroxidases. Four isoforms of Mn-dependent peroxidase and three of versatile peroxidase were obtained on native PAGE. By IEF separation, five isoforms of Mn-dependent peroxidase and only two of versatile peroxidase were observed. The results demonstrated that *G. lucidum* has potential for mineralization and transformation of various agricultural residues and should take more significant participation in large-scale biotechnological processes.

Key words: Ganoderma lucidum; Laccase; Mn-dependent peroxidase; Versatile peroxidase; Wheat straw

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INTRODUCTION

Species of the genus *Ganoderma* have taken an important place in various studies during the last few decades. These species produce numerous biologically active compounds (Berovič et al. 2003) and, therefore, they are attractive objects of medicinal and pharmaceutical researches. *Ganoderma* species are also often used in plant pathology investigations because they are pathogens of several arboreal species (Silva et al. 2005). Nowadays, their ligninolytic enzyme system presents an important area of interest, with the goal of introduction of the types and characteristics of produced enzymes, as well as the possibilities of their application in various biotechnological processes.

Ganoderma lucidum (Curt.: Fr.) Karst. synthesizes three extracellular ligninolytic enzymes: lacasse (EC 1.10.3.2), Mn-dependant peroxidase (EC 1.11.1.13), and lignin peroxidase (EC 1.11.1.14) (D`Souza et al. 1996, 1999; Silva et al. 2005; Varela et al. 2000). Due to the capability of producing the mentioned enzymes, *G. lucidum* is able to

modify and degrade various aromatic compounds, among which lignin takes a prominent place.

Physiological demands for ligninolytic enzymes production vary among white-rot species, and even among strains of a species. The factors that affect enzymes activity and type are: cultivation type (submerged or solid-state), carbon and nitrogen sources and concentrations, presence or absence of different inducers, medium pH, temperature, agitation, cultivation period, etc. (Silva et al. 2005; Stajić et al. 2006). Results of numerous studies have demonstrated that various agricultural and industrial residues are better substrates for enzymes production than glucose or other simple saccharides (Maltseva et al. 1991; Kapich et al. 2004; Moldes et al. 2004; Songulashvili et al. 2006). Wheat straw represents a very common agricultural residue worldwide, which contains a certain amount of soluble carbohydrates and inducers of enzyme synthesis (Morgan et al. 1993; Mckean and Jacobs 1997; Hofrichter et al. 1999), and therefore appears as a prospective substrate for bioconversion into fungal biomass and ligninolytic enzymes. Although several researches have shown that ligninolytic enzymes are produced during the secondary metabolism under conditions of limited nitrogen (Hammel 1997; Master and Field 1998), the nature and concentration of nitrogen sources are important factors of production regulation (Maltseva et al. 1991; Silva et al. 2005).

Whether nitrogen source and concentration could affect characteristics of selected *G. lucidum* ligninolytic enzymes, thereby also affecting wheat straw fermentation, was the question that provided the goal for the present study.

EXPERIMENTAL

Materials

The culture of *Ganoderma lucidum* HAI 447 was obtained from the culture collection of the Institute of Evolution, University of Haifa (Israel), and preserved on malt agar medium in the culture collection of the Institute of Botany, Faculty of Biology, University of Belgrade (Serbia).

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 (dH_2O); and *(iv)* biomass homogenization with 100 ml of sterile dH_2O in laboratory blender.

Solid-state fermentation was carried out at 25 ºC in 100 ml flasks containing 2 g of wheat straw as the carbon source, and 10 ml of the modified synthetic medium (without glucose, with one of the selected inorganic or organic nitrogen sources, and different pH). The used inorganic nitrogen sources were NH_4NO_3 and $(NH_4)_2SO_4$ in

nitrogen concentrations of 10, 15, 20, 25, 30, and 40 mM, and the organic source was peptone (total nitrogen content 14.1) in concentrations of 0.25, 0.5, 1.0, 2.0, and 4.0%. The medium composed of wheat straw and distilled water was used as the control. The analyzed initial pH values of the mentioned media were: 3.5, 4.0, 4.5, 5.0, and 6.0.

Suspension obtained after inoculum homogenization was used for inoculation (3 mL per flask). Samples from flasks were harvested after 7 days of cultivation, and the extracellular enzymes were extracted by stirring of samples with 50 mL distilled water using a magnetic stirrer for 10 min at 4ºC. The obtained extracts were separated by centrifugation (4ºC, 5000 rpm, 15 min), and supernatants were used for measurements of activities of laccase (Lac) and Mn-oxidizing peroxidases [Mn-dependent peroxidase (MnP) and versatile peroxidase (VP)], as well as total protein content. Three replications for each studied nitrogen source and concentration, as well as initial medium pH value, were performed.

Enzyme activity assays

Lac activity was assayed using 50 mM ABTS (ε_{436} = 29 300 $M^{-1}cm^{-1}$) as a substrate in 0.1 M phosphate buffer (pH 6.0). The reaction mixture contained buffer, ABTS, and sample (V_{tot} = 1 mL).

Activities of Mn-oxidizing peroxidases were measured with 3 mM phenol red (ε_{610} = 22 000 $M^{-1}cm^{-1}$) as a substrate in a buffer with the following content: succinic acid disodium salt, bovine serum albumin, and DL-lactic acid sodium salt (pH 4.5). The reaction mixture (V_{tot} = 1 mL) contained: buffer, sample, 2 mM H_2O_2, and phenol red, with or without 2 mM $MnSO_4$ (for MnP and VP, respectively). The reaction was stopped by 2 M NaOH.

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

Determination of total proteins

Determinations of the amounts of total proteins were performed according to the method of Silva et al. (2005). The reaction mixture contained 0.80 mL of the sample and 0.20 mL of Bradford's reagent, and absorbance was measured at 595 nm after reacting at room temperature for 5 min.

Electrophoresis

Protein patterns of 7-day-old solid-state *G. lucidum* culture extract from media enriched with studied nitrogen sources at the optimum nitrogen concentrations and pH for the enzymes activity were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS/PAGE). SDS/PAGE was performed on 12% polyacrylamide with a reservoir buffer consisting of 0.025 M TRIS and 0.192 M Gly (pH 8.3), at 24 mA for 120 min. Protein bands were visualized by silver staining. Molecular mass standard (Bio-Rad) was used.

Determination of Lac and Mn-oxidizing peroxidases activity was done by separation of protein extract by native PAGE and isoelectric focusing (IEF). Native electrophoresis was performed on 5% stacking and 10% running gel. IEF was carried out

in 7.5% polyacrylamide gel with 3% ampholyte on a pH gradient of 3 to 9. Markers for IEF of the pI range 3.6 to 9.3 were purchased from Sigma (IEF-M1A). Bands with Lac activity were located by the gel incubation in 10 mM ABTS in 200 mM phosphate buffer (pH 5.0). MnP and VP activities were detected by specific staining, using 10% 4-chloro-naphthol and 0.03% H_2O_2 in 100 mM K-phosphate buffer (pH 6.5) with or without 100 mM $MnSO_4$.

RESULTS AND DISCUSSION

The activities of Lac and Mn-oxidizing peroxidases as well as total protein content, in crude extract of *G. lucidum* HAI 447, varied depending on nitrogen sources and concentrations and the initial medium pH. An initial medium pH of 5.0 was mainly the optimum for production of analyzed enzymes and proteins.

The highest level of Lac activity was noted in NH_4NO_3-enriched medium, in a nitrogen concentration of 10 mM and an initial medium pH of 5.0 (181.4 ± 4.0 U/L), which was 2.4-fold higher compared to the control medium (76.1 ± 6.9 U/L) where wheat straw was the unique nitrogen source (Fig. 1). On the contrary, enrichment of the medium with another selected inorganic nitrogen source, $(NH_4)_2SO_4$, induced a decrease of Lac activity compared to the control, except in a nitrogen concentration of 15 mM and the initial medium pH of 5.0, where it was slightly higher (81.7 ± 0.7 U/L) (Fig. 1A). The presence of peptone at a concentration of 0.25% and an initial medium pH of 4.5 caused a maximum increase of Lac activity (136.2 ± 1.5 U/L), compared to the control. However, in a concentration of 0.5% activity rapidly decreased, even below the control level, and with further concentration increase, Lac activity slightly rose to the control level (Fig. 1B).

During submerged fermentation of wheat bran by *G. lucidum* HAI 447, Songulashvili et al. (2007) noted maximum Lac activity in the presence of KNO_3, at a nitrogen concentration of 10 mM, while NH_4NO_3 was less suitable nitrogen source (Lac activity was approximatelly twice less). Contrary to these results, organic nitrogen sources (especially peptone) were the best for Lac activity in numerous mushroom species, such as *Trametes gallica*, *T. trogii*, *T. villosa*, and *Pleurotus ostreatus* (Dong et al. 2005; Mikiashvili et al. 2006; Levin et al. 2010). The received data about the optimum nitrogen concentration for Lac activity were in accordance with previously obtained results, which showed that high nitrogen levels repressed ligninolytic enzymes production in *Phanerochaete chrysosporium*, *T. versicolor*, and *Pycnoporus cinnabarinus* (Buswell et al. 1984; Eriksson et al. 1990; Tekere et al. 2001). Moreover, contrary to these results, D`Souza et al. (1996, 1999) reported that Lac activity in the shaken cultures of studied *G. lucidum* strain was more than 4-fold higher in the medium with high-nitrogen content than in that with low-nitrogen amount. High levels of Lac activity were also noted during submerged cultivation of *Agaricus bisporus*, *Lentinula edodes*, *Cyathus stercoreus*, *P. eryngii*, *P. ostreatus, P. pulmonarius*, and one of *T. versicolor* strain in media with significant nitrogen amount (Perry et al. 1993; Buswell et al. 1995; Sethuraman et al. 1999; Galhaup et al. 2002; Stajić et al. 2006). Besides numerous results that demonstrated influence of nitrogen source and concentration on Lac activity, Li et al.

(2010) showed that nitrogen source did not have the significant influence on Lac activity in *G. lucidum*.

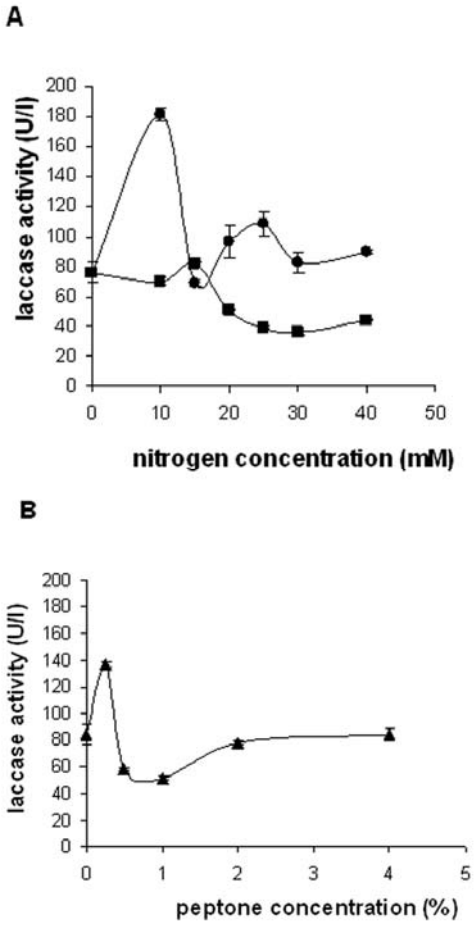

Fig. 1. Effect of selected nitrogen sources and concentrations, at the optimum initial medium pH, on laccase activity. **A** —●— NH_4NO_3; —■— $(NH_4)_2SO_4$; **B** —▲— peptone. (Data represent mean value of activities of three different samples. Variations are given as standard errors).

NH_4NO_3 was also the most appropriate nitrogen source for the MnP activity. The maximal activity level was noted at a nitrogen concentration of 20 mM and an initial medium pH of 5.0 (112.8 ± 1.0 U/L), when it was 7-fold higher than in the control medium (15.7 ± 0.8 U/L), while further increases of nitrogen concentration caused a gradual decrease of activity (Fig. 2A). At the nitrogen concentration of 20 mM, in the case of $(NH_4)_2SO_4$, and at the peptone concentration of 0.25% (initial medium pH 5.0), MnP activity was the most significant compared to the control (53.5 ± 1.6 U/L and 54.4 ± 2.6 U/L, respectively). However, at higher concentrations the activity level was decreased to values even below the control (Fig. 2B, C).

Production of VP was insignificant in all studied nitrogen sources and concentrations, as well as the initial medium pH (Fig. 2). The maximum activity level was noted in 0.25% peptone-enriched medium at initial pH 6.0 (35.0 ± 1.9 U/L), which was approximately 3-fold higher than in the control (12.5 ±0.7 U/L).

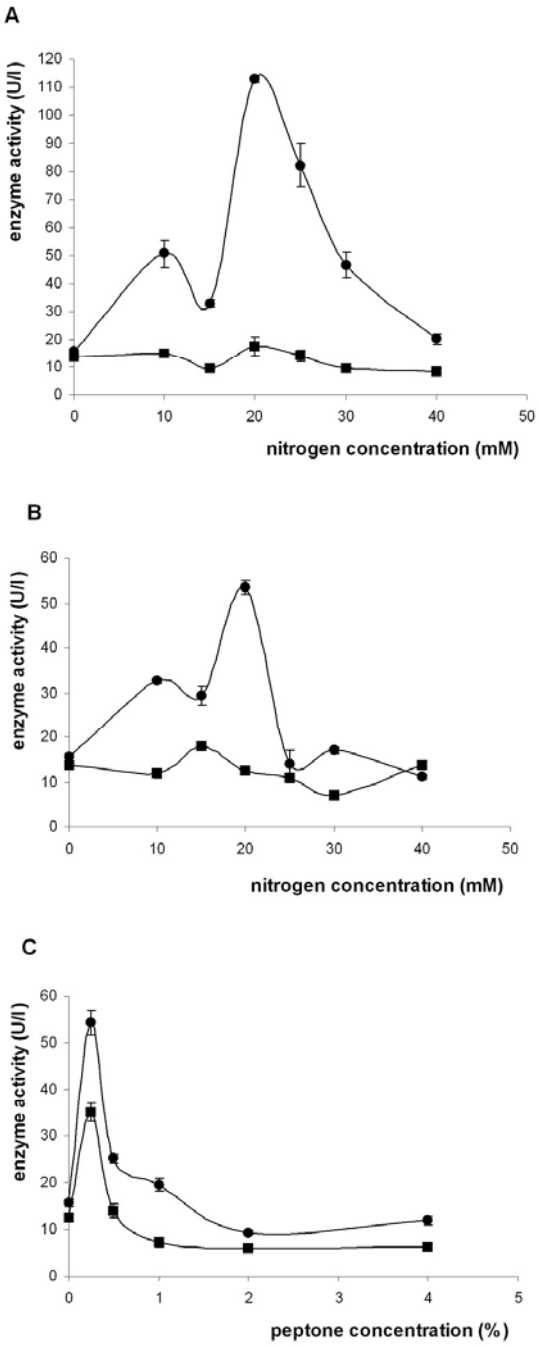

Fig. 2. Effect of selected nitrogen sources and concentrations, at the optimum initial medium pH, on Mn-oxidizing peroxidases activities. **A** NH_4NO_3; **B** $(NH_4)_2SO_4$; **C:** peptone. —●— Mn-dependent peroxidase; —■— versatile peroxidase. (Data represent mean value of activities of three different samples. Variations are given as standard errors.)

During submerged fermentation of wheat bran by *G. lucidum* HAI 447, NH_4NO_3 and $(NH_4)_2SO_4$ affected inhibitory to MnP activity compared to the control medium without any nitrogen source, and the highest value of the activity was noted in the presence of KNO_3 at a nitrogen concentration of 10 mM (Songulashvili et al. 2007). However, in other mushroom genera and species the organic nitrogen source (peptone) strongly improved MnP production (Kaal et al. 1995; Levin et al. 2010). The effect of nitrogen concentration on MnP activity varied, depending on the species. Thus, in *Cyathus stercoreus*, *Ganoderma adspersum*, and *Phellinus robustus* significant nitrogen concentration slightly repressed MnP activity compared to the control (Sethuraman et al. 1999; Songulashvili et al. 2006), while in *Pleurotus ostreatus* and *P. pulmonarius* activity of Mn-oxidizing peroxidases was stimulated by higher nitrogen concentrations (Stajić et al. 2006).

The total protein content rapidly decreased with the addition of any of the selected nitrogen sources and concentrations to the medium, except with NH_4NO_3, in a nitrogen concentration of 15 mM, and the initial pH of 4.5, when it was approximately the same as in the control medium (0.12 ± 0.01 mg/mL) (Fig. 3).

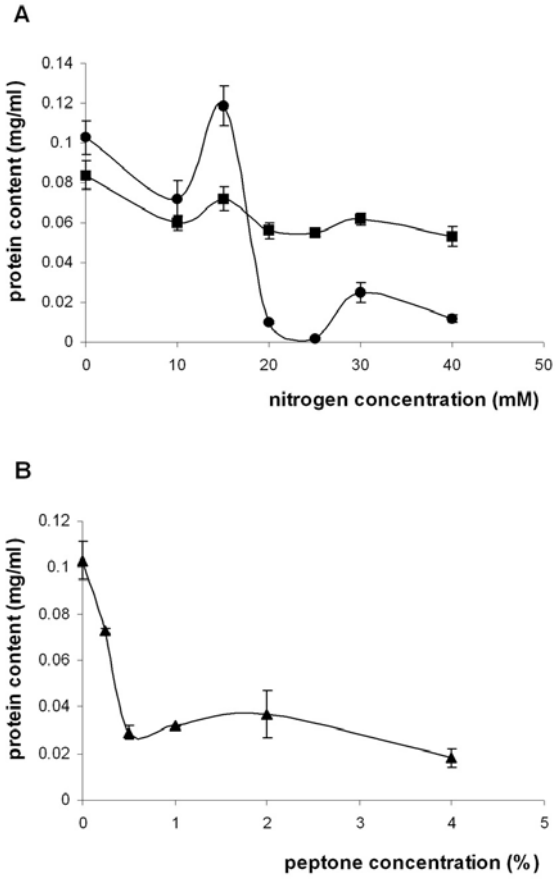

Fig. 3. Effect of selected nitrogen sources and concentrations, at the optimum initial medium pH, on total protein content. **A** —●— NH_4NO_3; —■— $(NH_4)_2SO_4$; **B** —▲— peptone. (Data represent mean value of protein content of three different samples. Variations are given as standard errors)

Songulashvili et al. (2007) obtained significantly higher protein production (ranging between 0.64 and 0.66 mg/mL) in the same *G. lucidum* strain after wheat bran submerged fermentation in the presence of NH_4NO_3, $(NH_4)_2SO_4$, and peptone (at the nitrogen concentration of 10 mM). The difference in the protein production could be explained primarily by differences in the type and period of cultivation.

Protein patterns from 7-day-old *G. lucidum* culture extract from solid-state wheat straw media enriched with the selected nitrogen sources at optimum nitrogen concentrations and initial medium pH for the enzymes production, after SDS/PAGE were similar to each other, showing significant differences in the intensity (Fig. 4). Unique differences were bands of molecular masses of 27 kDa and 43 kDa, which were clearly defined on the gel of extract from $(NH_4)_2SO_4$- and peptone-enriched medium.

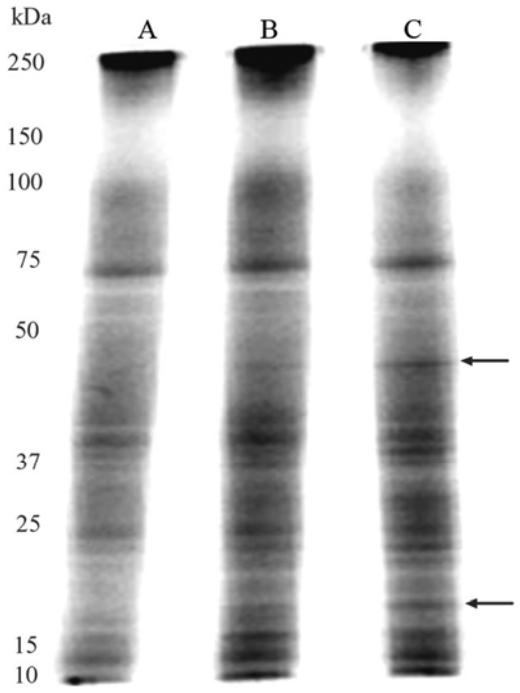

Fig. 4. SDS/PAGE of extracellular proteins produced by *Ganoderma lucidum* HAI 447 cultivated under solid state fermentation of wheat straw in the presence of: **A** NH_4NO_3; **B** $(NH_4)_2SO_4$; **C** peptone

Four bands with Lac activity were detected after native PAGE, three bands with higher electrophoretic mobility, and one weak band with lower electrophoretic mobility. Four Lac isoforms were obtained by IEF separation from NH_4NO_3- and $(NH_4)_2SO_4$-enriched medium, two with pI about 4.6 and two stronger bands with pI about 3.6 (Fig. 5). In peptone-enriched medium, only two Lac isoforms with considerably higher intensity than in the medium with one of the tested inorganic nitrogen sources and with pI about 3.6 were detected (Fig. 5).

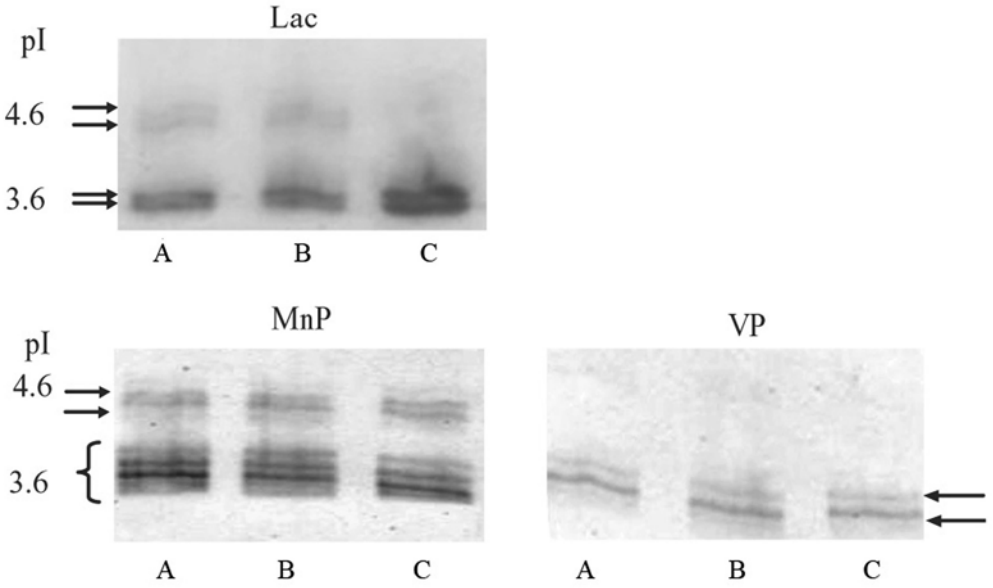

Fig. 5. Isoelectric focusing pattern of *Ganoderma lucidum* HAI 447 laccase (Lac), Mn-dependent peroxidase (MnP), and versatile peroxidase (VP): **A** NH_4NO_3; **B** $(NH_4)_2SO_4$; **C** peptone

Contrary to the obtained results, D`Souza et al. (1996) separated two Lac isoenzymes, *Gl*1 and *Gl*2, and characterized their genes during cultivation of *G. lucidum* in malt medium, while Ko et al. (2001) purified and characterized three Lac isoenzymes, GaLc1, 2, and 3, by cultivation of Korean *G. lucidum* strain in liquid glucose/ peptone/yeast extract medium. The mentioned results have shown that nitrogen sources play an important role not only at the level of Lac activity, but also in their isoforms profile. Dong et al. (2005) explained these results by influence of nitrogen source on isozyme gene expression.

Four isoforms of MnP and three of VP were obtained on native PAGE gels of all studied media extracts. However, five bands with MnP activity (three with pI 3.6 and two with pI 4.6), and only two weak bands with VP activity (with pI about 3.6) were separated by IEF of the mentioned culture extracts (Fig. 5). The intensity of bands with MnP activity were different depending on the nitrogen source added to the medium, while VP bands had approximately the same intensity. MnP bands obtained by IEF separation of extract from $(NH_4)_2SO_4$-enriched medium were weaker than those revealed from NH_4NO_3- or peptone-enriched medium (Fig. 5).

Although numerous studies of different white-rot mushroom species showed significant participation of Mn-oxidizing peroxidases (especially MnP) in lignin degradation, data on their isoforms profile in *G. lucidum* have not been reported until now. This is also the first study reporting the presence of VP activity in crude extract of *G. lucidum* culture.

This type of study is significant because recently special attention in biotechnology has been given to obtaining large amounts of low-cost enzymes by usage of

various agricultural and food industry residues, which can often be serious environmental pollutants. The residues present excellent substrates for fungal growth, which are mineralized to low-molecular weight compounds by various lignocellulolytic enzymes. These low-molecular weight products are easily absorbed by fungi, better digested by animals, and could be used in further processing such as in producing food of high nutrition value (mushroom fruiting bodies), feeds, and basic commodities for different industrial purposes.

CONCLUSIONS

1. This is the first study reporting the presence of versatile peroxidase activity in the crude extract of *G. lucidum* culture, as well as isoforms profile of Mn-oxidizing peroxidases.
2. An initial medium pH of 5.0 was generally the optimum for production of laccase, Mn-oxidizing peroxidases, and proteins.
3. The optimum nitrogen source for laccase and Mn-dependent peroxidase activity was NH_4NO_3, while peptone was the optimum one for versatile peroxidase activity.
4. Four laccase isoforms were obtained by native PAGE and IEF separations from NH_4NO_3– and $(NH_4)_2SO_4$–enriched medium, and only two bands from pepton-enriched medium.
5. Medium composition was not shown to affect isoenzyme patterns of Mn-oxidizing peroxidases.
6. Four isoforms of Mn-dependent peroxidase and three of versatile peroxidase were obtained on native PAGE, while on IEF gels their number was five and two, respectively.

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