Intraspecific Diversity in the Production and Characterization of Laccase within *Ganoderma lucidum*

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Ganoderma lucidum has a well-developed ligninolytic enzyme system, where laccase is the dominant and sometimes only synthesizing enzyme, and therefore could find an application in the delignification of abundant plant raw materials and in food, feed, paper, and biofuel production. The questions that provided the goals for the present study were whether the profile of *G. lucidum* laccase depends on cultivation type and carbon source, as well as whether intraspecific diversity exists. Conditions of submerged cultivation proved more preferable for laccase activity compared with solid-state cultivations in all studied strains, while oak sawdust provided a better carbon source than wheat straw. Maximum laccase activity (7241.0 U/L) was measured on day 14 of oak sawdust submerged fermentation by strain BEOFB 431. Intraspecific diversity in synthesized proteins was more significant in wheat straw than in oak sawdust submerged fermentation. The profile of laccase isoforms was dependent on strain, plant residue, type, and period of cultivation. Four acidic laccase isoforms (pI 3.6) were detected in *G. lucidum* BEOFB 431 at the same cultivation point where maximal enzyme activity was measured.

Keywords: Fermentation; Ganoderma lucidum; Laccase; Oak sawdust; Wheat straw

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INTRODUCTION

Agricultural and industrial expansion leads to the production of enormous amounts of agricultural, forest, industrial, and domestic wastes, all of which could be serious environmental pollutions. The annual production of various plant raw materials reaches approximately 170 to 200 billion tons, of which only 3% is used directly. The rest becomes environmental ballast because of its relatively slow degradation due to high lignin and low nitrogen contents (Zechendorf 1999; Pahkala and Pihala 2000; Dias *et al.* 2010). However, plant biomass as well as other frequent wastes could present potential resources for food, feed, paper, and energy production (Pahkala and Pihala 2000; Tabka *et al.* 2006; Yusoff 2006; Rodriguez *et al.* 2008). Mushroom fruiting bodies, an excellent source of proteins, are a high nutritionally valued food that is produced in this way. Current steady world population growth has resulted in a lack of food, 870 million malnourished people, and 10 million deaths every year (FAO 2012). Additionally, if it is taken into account that malnutrition is in positive correlation with protein deficit, slow child growth rate, and the appearance of various kinds of infection (Caulfield *et al.* 2004), then it is apparent that the importance of mushroom production and usage in the diet are high. The degradation of lignin and the breaking down of the hemicellulose-lignin matrix

from plant residues increase their digestibility by ruminants and therefore their potential for utilization as animal feed (Sharma and Arora 2010). Agricultural crop residues could also be alternative sources for paper production, which would protect the autochthonous forest (González *et al.* 2009). Currently, 80% of the primary energy consumed in the world is obtained from fossil fuels, which have negative effects on both the environment and the global economy (Nigam and Singh 2011). Therefore, special attention has been given to the consumption of renewable and biodegradable energy sources such as biofuels, hydrogen, and natural gas (Elshahed 2010). However, the transformation of the plant waste involves a complex degradation process whose efficiency depends on the potential of the organism-degrader, oxidative mechanisms, and culture conditions (Wan and Li 2010). Could mushroom species play a role in the process? Mushrooms, especially white-rot species, are one of the main degraders of plant raw materials because they possess a ligninolytic enzyme system. The current trend is to screen the capacity of new species and strains for producing these enzymes.

Ganoderma lucidum (Curt.: Fr.) Karst., as a white-rot species, synthesizes lacasses (EC 1.10.3.2), Mn-oxidizing peroxidases (EC 1.11.1.13), and lignin peroxidases (EC 1.11.1.14) (D'Souza *et al.* 1996; 1999; Varela *et al.* 2000; Ko *et al.* 2001; Silva *et al.* 2005; Stajić *et al.* 2004). Because it is capable of producing the aforementioned enzymes, *G. lucidum* can modify and degrade various aromatic compounds, especially lignin, and can participate in various biotechnological processes.

Laccase is the dominant, and sometimes only, ligninolytic enzyme synthesized by *G. lucidum* that has a significant physiological and biochemical role (D'Souza *et al.* 1999; Ko *et al.* 2001). Therefore, the questions that provided the goals for the present study were whether the profile of *G. lucidum* laccase depends on cultivation type and carbon source, as well as whether intraspecific diversity exists.

EXPERIMENTAL

Materials

The cultures of studied *G. lucidum* strains were obtained from fruiting bodies from Serbia, Montenegro, and China (Table 1), and maintained on a malt agar medium in the culture collection of the Institute of Botany, Faculty of Biology, University of Belgrade (BEOFB).

Table 1. Studied *Ganoderma lucidum* Strains

Code of strain	Origin of strain
BEOFB 431	Bojčin forest, Belgrade, Serbia, from *Quercus* sp.
BEOFB 432	Chinese commercial strain
BEOFB 433	Košutnjak, Belgrade, Serbia
BEOFB 434	Igalo, Montenegro, from *Platanus orientalis*

Methods

Growth conditions

The inoculum preparation involved the following steps: *(i)* inoculation of 100.0 mL of the 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 100.0 mL of sterile dH_2O in a laboratory blender.

Solid-state cultivation was carried out at 25 ºC in 100-mL flasks containing 5.0 g of wheat straw or oak sawdust as the carbon source and 10.0 mL of the modified synthetic medium (without glucose). Submerged fermentation was carried out in 250-mL flasks with 50.0 mL of the modified synthetic medium enriched with 5.0 g of the ground tested plant raw materials, at room temperature on a rotary shaker. Suspension obtained after inoculum homogenization was used for medium inoculation (3.0 mL per flask for solid-state and 5.0 mL for submerged fermentation). Samples from the flasks were harvested after 7, 10, and 14 days of cultivation, with the aim of following the dynamic of enzyme activity. The extracellular enzymes produced during solid-state cultivation were extracted by sample stirring with 50.0 mL dH_2O at a magnetic stirrer at 4 ºC for 10 min, and the extracts were separated by centrifugation (at 4 ºC and 5000 rpm, for 15 min). After submerged cultivation, the samples were prepared by centrifugation. The obtained supernatants were used for the determination of laccase activity and total protein content. Three replications were performed for each sample.

Enzyme activity assays

Activity of laccase was defined by monitoring the A_{436} change related to the rate of oxidation of 50 mM 2,2'-azino-bis-[3-ethyltiazoline-6-sulfonate] (ABTS) (ε_{436} = 29300 M^{-1} cm^{-1}) in 0.1 M phosphate buffer (pH 6.0) at 35 ºC (Stajić *et al.* 2004). The reaction mixture contained buffer, ABTS, and sample (V_{tot} = 1.0 mL).

Enzymatic activity of 1 U was defined as the amount of enzyme that transforms 1 μmol of substrate per min. A BioQuest CECIL CE2501 spectrophotometer (England) was used for these assays.

Determination of total proteins

The amount of total proteins was determined using the Bradford method, using bovine serum albumin as the standard (Silva *et al.* 2005). The total protein content is presented as mg/mL.

Electrophoresis

Screening of the laccase profile was performed for all analyzed *G. lucidum* strains in the optimum type and period of fermentation of both tested plant raw materials, as well as for the best laccase producer under all cultivation conditions.

Samples for SDS-PAGE were centrifuged (at 20 ºC and 13000 rpm, for 3 min) and together with buffer [0.5 M Tris HCl (pH 6.8), 85% glycerol, SDS, 0.1% brom phenol blue, and β-merkaptoetanol (ß-ME)] were heated in boiling water for 3 min. This type of electrophoresis was performed on 4% stacking and 10% running polyacrylamide gels in buffer with the following contents: 0.025 M Tris, 0.192 M Gly, 0.1% SDS (pH

8.3), at 80 V through concentrated and 120 to 160 V through separated gel for 120 min. Completed electrophoresis gels were fixed in a solution that contained 50% methanol, 10% acetic acid, and dH_2O to 100%, and then protein bands were visualized by staining with Coomassie Briliant Blue-G (CBB G). Molecular mass standard of a wide range (6.5 to 200.0 kDa (Sigma-Aldrich)) was used.

Determination of laccase isoforms was performed by separating protein extract by native-PAGE and defining isoelectric point (pI) by isoelectric focusing (IEF). Samples for native-PAGE were mixed with buffer of the same content as for SDS-PAGE but without SDS and ß-ME and without heating. Native electrophoresis was performed on 4% stacking and 10% running gel. Bands with laccase activity were located by the gel incubation in 10 mM ABTS in 200 mM phosphate buffer (pH 5.0). IEF was carried out in 7.5% polyacrylamide gel with 5% ampholyte on a pH gradient from 3.0 to 10.0 in three phases: *(i)* at 100 V for 15 minutes; *(ii)* at 200 V for 15 min; and *(iii)* at 450 V for 60 min. Completed focusing gel was fixed in trichloroacetic acid and protein bands were detected by staining using CBB G. IEF marker in a pI range of 3.6 to 9.3 (Sigma-Aldrich) was used.

RESULTS AND DISCUSSION

Laccase activity was detected after both the submerged and solid-state fermentation of wheat straw and oak sawdust at all measurement points (Fig. 1). Generally, submerged cultivation was the more appropriate cultivation type for laccase activity compared with solid-state cultivation, and oak sawdust was a better carbon source than wheat straw.

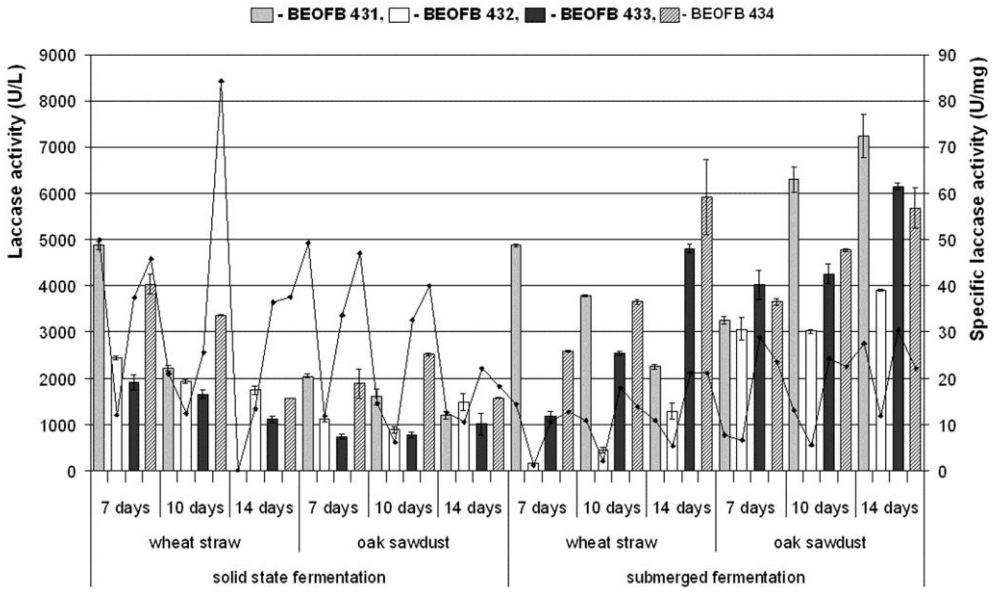

Fig. 1. The laccase activity of *Ganoderma lucidum* strains depending on type and period of wheat straw and oak sawdust fermentation

The peak laccase activity was observed after 14 days of oak sawdust submerged fermentation by *G. lucidum* BEOFB 431 (7241.0 ± 468.0 U/L), which was almost 3-fold higher than the maximum value obtained after 10 days of solid-state fermentation of the same plant residue by strain BEOFB 434 (2517.0 ± 21.0 U/L). The differences among minimum values of laccase activity obtained after oak sawdust fermentation by tested strains were more significant, from 742.5 ± 49.0 U/L (after 7 days of BEOFB 433 solid-state cultivation) to 3020.5 ± 41.5 U/L (in strain BEOFB 432 after 10-day-old submerged cultivation). Increased laccase activity during oak sawdust submerged fermentation were observed in all studied *G. lucidum* strains, which was not noted in solid-state cultivation (Fig. 1). However, under conditions of solid-state fermentation, a gradual decrease of laccase activity during the cultivation period was found in *G. lucidum* BEOFB 431, contrary to strain BEOFB 433, where activity slighty increased. In strain BEOFB 432, activity level declined on day 10 and rose on day 14, and in BEOFB 434 the peak of activity was noted on day 10 and the minimum on day 14 (Fig. 1).

Significant laccase activity was also noted after wheat straw fermentation, especially after submerged fermentation. Thus, the highest enzyme level was observed on day 14 of *G. lucidum* BEOFB 434 cultivation (5921.5 ± 813.0 U/L), while the strain BEOFB 431 was the best laccase producer during the solid-state fermentation of this residue (4872.0 ± 93.5 U/L, on day 7). According to the dynamic of laccase activity during wheat straw submerged fermentation, studied *G. lucidum* strains could be classified as one of two groups: *(i)* activity decreases with cultivation period (strain BEOFB 431); and *(ii)* level of laccase activity gradually increases from day 7 to day 14 of cultivation (strains BEOFB 432, BEOFB 433, and BEOFB 434). During the solid-state cultivation of wheat straw, a decrease in laccase activity was detected in all studied strains, especially in BEOFB 431, where on day 14 no activity was detected (Fig. 1).

The total protein content was also higher after submerged cultivation of the strains. Thus, the peak amount was noted after 10 days of submerged fermentation of oak sawdust by *G. lucidum* BEOFB 432 (548.0 ± 23.0 mg/mL), while the minimum production was observed after the solid-state cultivation of BEOFB 433 and BEOFB 434 on both oak sawdust and wheat straw. This profile of protein production reflected specific laccase activity (Fig. 1).

Protein patterns after SDS/PAGE revealed the existence of significant intraspecific diversity, as well as differences in the same strain depending on plant residue (Fig. 2). A higher level of differences among strains was obtained after submerged cultivation in wheat straw-enriched medium. Thus, a band of 29 kDa was clearly separated from the extracts of *G. lucidum* BEOFB 432, BEOFB 433, and BEOFB 434, while a band of 97 kDa was noted in strains BEOFB 433 and BEOFB 434. In strain BEOFB 431, one strong band of 40 kDa as well as three weak bands of molecular masses in the range of 66 kDa to 97 kDa were visualized (Fig. 2A). However, after submerged fermentation of oak sawdust, the level of similarity in protein band arrangement among strains was more significant. The bands of molecular masses of 14, 36, 40, and 66 kDa were noted in all studied strains, and a band of 29 kDa was observed in all except strain BEOFB 431 (Fig. 2B).

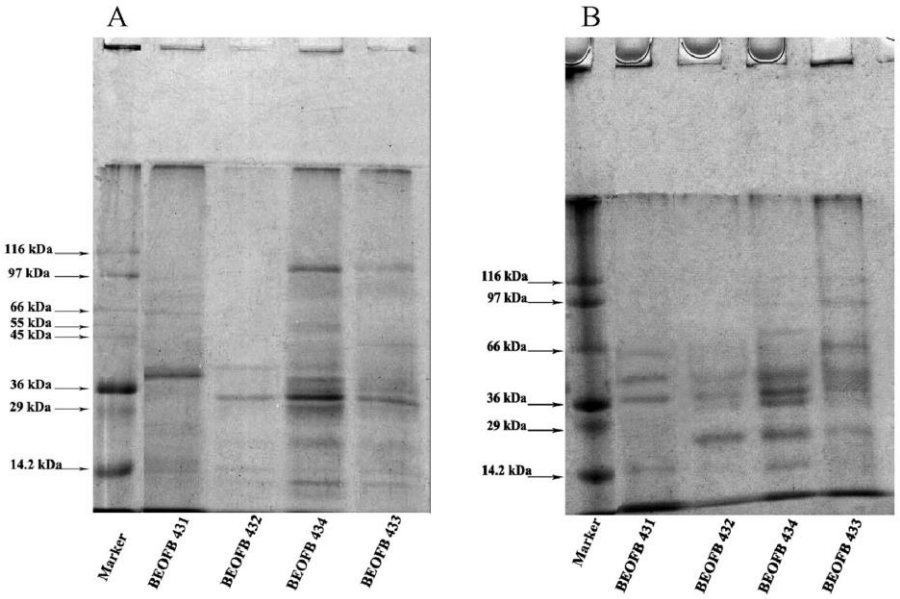

Fig. 2. SDS/PAGE of extracellular proteins produced by *Ganoderma lucidum* strains after 14 days of submerged fermentation of (A) wheat straw and (B) oak sawdust

Laccase isoforms detected in tested *G. lucidum* strains were more numerous after oak sawdust submerged fermentation. Thus, at the end of 14 days of cultivation in the wheat straw-enriched medium, three bands were separated in strain BEOFB 431 and only one in the other strains (Fig. 3A). Under the same conditions of oak sawdust fermentation, four laccase isoforms were observed in strain BEOFB 431, compared with only two in the other strains (Fig. 3B).

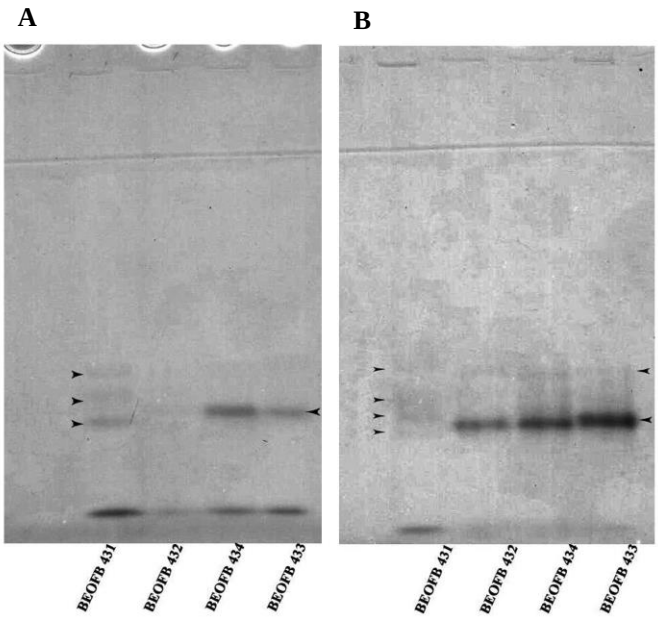

Fig. 3. Native/PAGE patterns of laccases in studied *Ganoderma lucidum* strains after 14 days of submerged fermentation of (A) wheat straw and (B) oak sawdust

The profile of laccase isoforms in the best producer (strain BEOFB 431) changed during the cultivation period and was also dependent on cultivation type (Fig. 4). Thus, during submerged cultivation of the strain in wheat straw-enriched medium, no laccase band was detected after 7 and 10 days, while on day 14, three bands were visible (Fig. 4A). After 7 days of wheat straw solid-state fermentation by this strain, two laccase bands were separated and with cultivation extention that number decreased to only one band (Fig. 4A). During oak sawdust solid-state fermentation, three isoforms were noted on days 7 and 10 and only one after 14 days, while on day 14 of submerged cultivation, four isoforms were visible (Fig. 4B). All separated laccase isoforms of strain BEOFB 431 were acid, with a pH of 3.6.

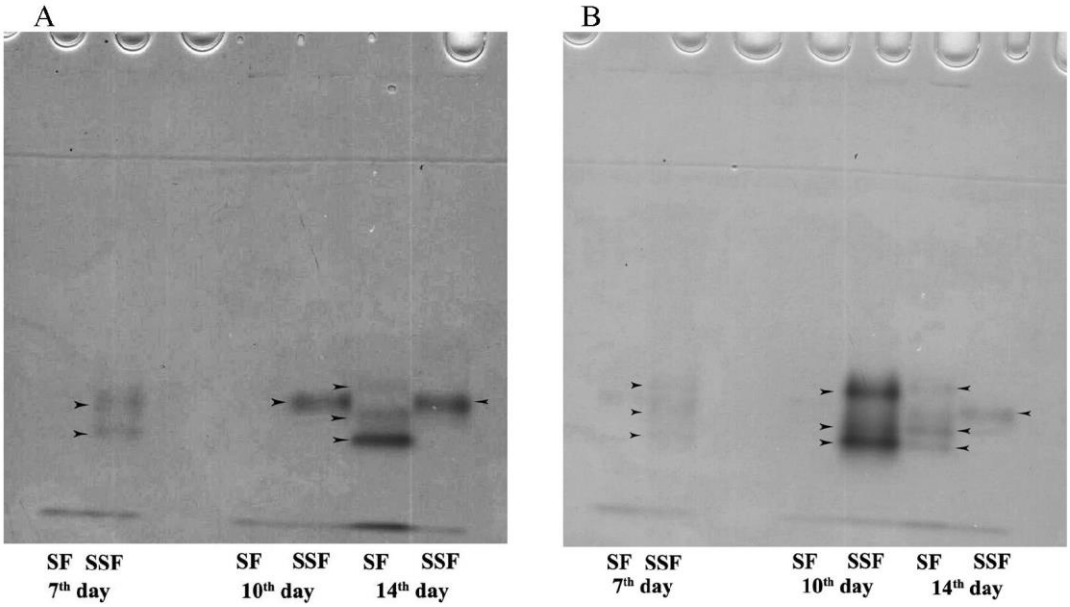

Fig. 4. Native/PAGE temporal profile of *Ganoderma lucidum* BEOFB 431 laccase after solid-state and submerged fermentation of wheat straw (A) and oak sawdust (B)

Considering the significant proportion of lignin (between 23% and 30%) in wheat straw and oak sawdust (Nishimura and Matsuyama 1989; Rakić *et al.* 2006; Salvachúa *et al.* 2011), efficient exploitation of these plants' raw materials requires pretreatment by lignin degraders, among which mushrooms hold an important place. A degrader's effectiveness depends on its capacity to produce ligninolytic enzymes, which is affected by lignin content in relation to hemicellulose and cellulose, as well as by cultivation method (D'Souza *et al.* 1999; Arora and Gill 2000; Fenice *et al.* 2003; Moldes *et al.* 2004; Songulashvili *et al.* 2006). Numerous studies show the existence of significant inter- and intraspecific mushroom diversity in enzyme activity. Thus, Stajić *et al.* (2004) and Songulashvili *et al.* (2007) reported significant differences in enzyme activity among 11 *Pleurotus* species and between two *Ganoderma* species, respectively, during both submerged and solid-state fermentation of selected plant residues. However, differences in laccase activities were also noted at an intraspecific level within *Cerrena maxima*, *P. ostreatus*, *P. pulmonarius*, *P. eryngii*, *Trametes versicolor*, and *G. lucidum* (Camarero *et al.* 1996; Stajić *et al.* 2004; Silva *et al.* 2005; Songulashvili *et al.* 2007; Simonić *et al.* 2010).

The effect of plant residue chemical composition on laccase production and activity has been demonstrated by numerous previous studies. Thus, Arora and Gill (2000) showed that the enzyme level was higher during sugar cane fermentation than during wheat and rice straw fermentation by *Phlebia floridensis*. Olive mill wastewater stimulated enzyme production in *Panus tigrinus* (Fenice *et al.* 2003), while mandarin peels increased laccase activity in *G. adspersum* and *T. hirsuta* (Songulashvili *et al.* 2006; Ćilerdžić *et al.* 2011; Songulashvili *et al.* 2006).

The production and activity of ligninolytic enzymes and the level of lignin degradation also depend on cultivation type. Generally, submerged cultivation is preferred for the synthesis of hydrolases and laccase, while solid-state cultivation is the optimum for Mn-oxidizing peroxidase production (Elisashvili *et al.* 2008). The same authors demonstrated that laccase activity in *Coriolopsis polyzona* was 15-fold higher after beech leaves submerged fermentation than after solid-state fermentation. In *G. applanatum*, the obtained value was about 4-fold higher after submerged cultivation in a mandarine peels-enriched medium than after the solid-state fermentation of wheat bran (Elisashvili *et al.* 2009). In further studies, the same authors reported significant laccase production by *G. lucidum* IBB 447 submerged cultivation in wheat bran-enriched medium, which was explained by the fact that soluble hydrocarbons, aromatic compounds, and trace elements of the residues release into the medium and stimulate synthesis of the enzyme (Elisashvili *et al.* 2010).

The number of laccase isoforms visible in this study was the same or higher than those previously obtained, and depends on strain and cultivation conditions. Thus, D`Souza *et al.* (1996) separated two isoenzymes (*Gl*1 and *Gl*2) in *G. lucidum* and characterized their genes during cultivation in malt medium. Three isoforms (GaLc1, 2, and 3) were purified and characterized after submerged cultivation of the Korean strain *G. lucidum* in glucose/peptone/yeast extract medium (Ko *et al.* 2001). Certain of the components of a cultivation medium can selectively stimulate some isoforms in *G. lucidum*. Thus, Coelho *et al.* (2010) showed that the herbicides bentazone and diuron acted as inducers of lac2 and repressors of lac1 in *G. lucidum* compared with control glucose liquid media.

Noted differences in the laccase activity and isoenzyme profile could be explained by the fact that *G. lucidum* presents a worldwide distributed species-complex in which each strain is characterized by different physiological state and anabolic demands caused possibly by geographical origin. Based on the data it can be concluded that the strains have different capacity to degrade various plant raw material, which is a basis for their potential application in preprocessing of abundant agricultural and forest residues that could be good resources for production of fruiting body of medicinal important species, as well as fiber and energy.

A further contribution of the study is the demontration that submerged cultivation is more suitable cultivation type because it is a less time-consumable and more controllable way for obtaining a large amounts of low-cost laccases required for various biotechnological processes.

CONCLUSIONS

1. Intraspecific diversity in laccase properties within *Ganoderma lucidum* was noted.
2. Oak sawdust was a better substrate for laccase production than wheat straw.
3. Submerged cultivation was preferable for laccase production compared with solid-state fermentation.
4. The highest laccase activity was noted on day 14 of sawdust submerged fermentation.
5. Four acidic laccase isoforms were observed after oak sawdust submerged fermentation.

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