Biochemistry and Biophysics Reports 29 (2022) 101204

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

Relationship between fruiting body development and extracellular laccase production in the edible mushroom *Flammulina velutipes*

Aylin Cesur [a], Ryousuke Yamamoto [b], Yasuhiko Asada [b], Akira Watanabe [b,*]

[a] *Department of Applied Bioresource Science, The United Graduate School of Agricultural Sciences, Ehime University, 3-5-7 Tarumi, Matsuyama, Ehime, 790–8566, Japan*

[b] *Department of Applied Biological Science, Faculty of Agriculture, Kagawa University, 2393 Ikenobe, Miki-cho, Kagawa, 761–0795, Japan*

ARTICLE INFO

Keywords:
Flammulina velutipes
Fruiting body
Laccase

ABSTRACT

The biochemical mechanism underlying the development of fruiting bodies in *Flammulina velutipes*, an edible mushroom, was investigated using the YBLB colorimetric assay to distinguish between the normal strain (FVN-1) and the degenerate strain (FVD-1). In this assay, the color of the YBLB medium (blue-green) inoculated with FVN-1 exhibiting normal fruiting body development changed to yellow, while the color of the medium inoculated with FVD-1 changed to blue. In this study, we found that this color difference originated from extracellular laccase produced by FVN-1. Moreover, FVN-1 exhibited considerably higher extracellular laccase activity than FVD-1, under conditions facilitating fruiting body formation. Overall, these findings suggest that extracellular laccase is involved in the fruiting body development process in *F. velutipes*.

1. Introduction

Basidiomycetes are unique mushrooms that undergo dramatic morphological changes from vegetative mycelia to fruiting bodies [1–3]. Furthermore, basidiomycete fruiting bodies are used as food and source materials for biological response modifiers [4,5]. In line with this, the biochemical mechanism underlying fruiting body formation is an important research topic from both scientific and commercial perspectives. However, this biochemical mechanism is still unclear, due to the complexity of fruiting body development.

Flammulina velutipes, also known as Enokitake, is a commercially important cultivated edible basidiomycete mushroom, especially in Japan. Degenerate strains of *F. velutipes* (i.e., strains exhibiting the loss of fruiting body formation) have been reported since the 1980s [6]. The prevalence of degenerate strains has led to substantial financial losses in the mushroom industry. To resolve this issue, Magae *et al.* [6] established a simple colorimetric method, known as the YBLB assay, to distinguish between the normal *F. velutipes* strain (FVN-1) (Fig. 1A) and the degenerate strain (FVD-1) (Fig. 1B). Briefly, when the FVN-1 mycelium was grown in the YBLB medium, the medium exhibited a marked color change from blue-green to yellow (Fig. 1C), whereas FVD-1 caused a slight change in color from blue-green to blue (Fig. 1D). However, little is known about the biochemical mechanisms underlying this color change in the YBLB assay. Identification of the factors underlying the different responses between the FVN-1 and FVD-1 strains in the YBLB assay should contribute to a better understanding of the biochemical mechanism of fruiting body development not only in *F. velutipes*, but also in other basidiomycete mushrooms.

In this study, we examined the causal factor for this color change, and the results provide new insights into the biochemical mechanisms involved in *F. velutipes* fruiting body development.

2. Materials and methods

2.1. *F. velutipes* strains and culture conditions

Dikaryotic FVN-1 and dikaryotic FVD-1 were kindly provided by Dr. Yumi Magae. These strains were cultivated on potato dextrose agar (PDA, BD, Franklin Lakes, NJ, USA) plates for approximately 1 week at 23 °C. The samples were stored at 4 °C until further use. For fruiting body formation, sawdust medium containing 1.5 g sawdust, 1.5 g rice bran, and 5 mL ultrapure water in a small vial (27 × 55 mm; Nichiden-Rika Glass, Kobe, Japan) was inoculated independently with two mycelial plugs (7 mm diameter) of FVN-1 and FVD-1 grown on PDA

Abbreviation: FVN-1, normal *Flammulina velutipes* strain; FVD-1, degenerate *Flammulina velutipes* strain; PDA, potato dextrose agar; BTB, bromothymol blue; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid).

* Corresponding author.
E-mail address: watanabe.akira@kagawa-u.ac.jp (A. Watanabe).

https://doi.org/10.1016/j.bbrep.2022.101204
Received 4 November 2021; Received in revised form 4 January 2022; Accepted 5 January 2022
2405-5808/© 2022 Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

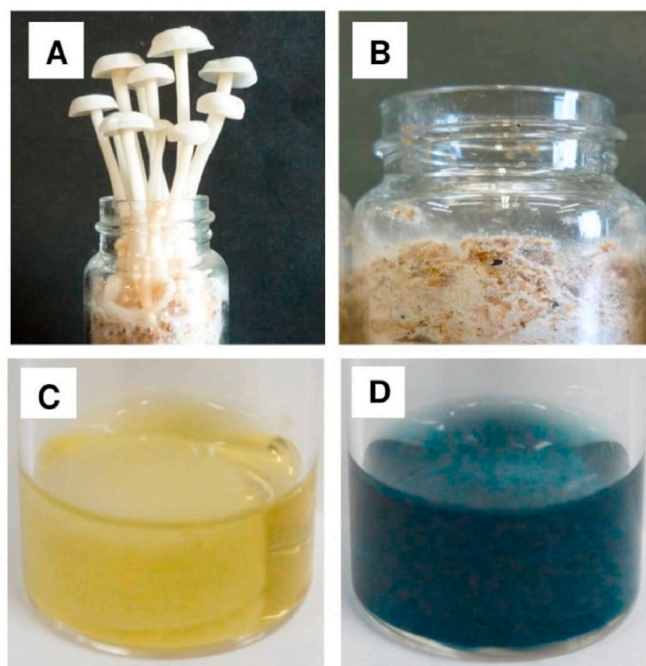

Fig. 1. Differences between normal (FVN-1) and degenerate (FVD-1) strains of *F. velutipes* in fruiting body development and YBLB assay results.
Fruiting body development was examined in FVN-1 (A) and FVD-1 (B) using the conventional sawdust medium. The YBLB assay of FVN-1 (C) and FVD-1 (D) was conducted by cultivating these strains in YBLB medium for 12 days.

plates. After the cultivation at 23 °C for two weeks in the dark, the culture was shifted to low temperature (15 °C) with continuous light to facilitate fruiting.

2.2. YBLB assay

The YBLB assay [6] was performed as reported previously, with a slight modification to increase the detection sensitivity. The YBLB medium was composed of 0.45% yeast extract (Nacalai Tesque, Kyoto, Japan), 0.75% Bacto Peptone (BD), 0.5% lactose monohydrate (Fujifilm Wako, Osaka, Japan), 0.0025% bromothymol blue (BTB, Nacalai Tesque), and 0.0001% $CuSO_4$ (Fujifilm Wako). The assay was carried out in a small vial (27 × 55 mm; Nichiden-Rika Glass) containing 5 mL of the medium. Each vial was inoculated with a piece of the mycelium (7 mm in diameter) of FVN-1 and FVD-1 grown on PDA plates. Cultures were incubated for 12 days at 23 °C in the dark with gentle shaking.

2.3. Enzyme assays

The activities of laccase, lignin peroxidase, and manganese peroxidase were measured spectrophotometrically. Laccase activity was assayed by measuring the oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS, Fujifilm Wako) with the increase in absorbance at 420 nm ($\mathcal{E}_{420} = 36{,}000$ M^{-1} cm^{-1}). The reaction mixture contained 100 mM sodium citrate buffer (pH 3.0), 1 mM ABTS, and an appropriate amount of enzyme in a final volume of 1.0 mL. One unit of laccase activity (U) was expressed as the amount of enzyme required to oxidize 1 μmol substrate per min at 30 °C. Lignin peroxidase [7] and manganese peroxidase [8] activity levels were measured according to previously reported methods.

2.4. Spectrophotometric measurements

Absorption spectra were obtained using a U-2010 spectrophotometer (Hitachi, Tokyo, Japan).

3. Results and discussion

3.1. Time course of pH and absorbance at 615 nm during the growth of F. velutipes strains in YBLB medium

The YBLB assay is based on the difference in color change between the FVN-1 and FVD-1 strains during their growth in the YBLB medium. As the YBLB medium contained BTB, a colorimetric pH indicator, we inoculated the FVN-1 and FVD-1 strains into the YBLB medium and measured the pH values of the media during growth (Fig. 2A). The pH values of media inoculated with both FVN-1 and FVD-1 strains exhibited a slight alkaline shift during the incubation period, indicating that the difference between normal and degenerate strains in the YBLB assay cannot be explained by the pH shift of the culture media.

To quantify the color change of the YBLB medium during the growth of FVN-1 and FVD-1, the maximum visible absorbance of BTB at 615 nm was measured. The absorbance of the uninoculated YBLB medium remained constant. The absorbance decreased gradually over 2 days in the YBLB medium inoculated with FVN-1 (Fig. 2B) and reached a minimum value (blue-green to yellow). In the case of FVD-1, BTB absorbance increased starting from the 2-day-old culture (blue-green to blue). This increase in absorbance can be attributed to the alkaline shift in medium pH during the growth of FVD-1. However, the color change in the YBLB medium during FVN-1 growth was probably caused by other factors, such as the modification of BTB by extracellular enzyme(s) produced by FVN-1, but not by FVD-1.

3.2. Laccase, lignin peroxidase, and manganese peroxidase activities during the growth of F. velutipes strains in YBLB medium

F. velutipes is a lignin-degrading mushroom. Generally, lignin-degrading fungi produce phenol-oxidizing enzymes, such as laccase, lignin peroxidase, and manganese peroxidase, which mediate the oxidative process of lignin degradation [9]. Such oxidizing enzymes are also involved in the decoloration (or discoloration) of various aromatic dyes [10–16]. Therefore, we analyzed the activity of these enzymes in the YBLB medium during the growth of the FVN-1 and FVD-1 strains of *F. velutipes*. As shown in Fig. 2C, laccase activity increased during the 4-day incubation in YBLB medium inoculated with the FVN-1 strain. With the FVD-1 strain, only a slight increase in laccase activity was detected throughout the incubation period, indicating considerably reduced laccase production compared to the FVN-1 strain. Neither lignin peroxidase nor manganese peroxidase activity was found in the media inoculated with both strains. These results strongly suggest that the color difference in the YBLB assay can be attributed to the difference in extracellular laccase between these strains. In other words, laccase alters the color of YBLB medium inoculated with FVN-1 from blue-green to yellow. To confirm that laccase changed the color of the YBLB medium from blue-green to yellow (as observed with the normal strain), we compared the absorption spectra of YBLB medium incubated with the commercial laccase from *Trametes versicolor* purchased from Jena Bioscience (Jena, Germany) and the medium incubated with the culture filtrate of the FVN-1 strain (12-day-old culture). As shown in Fig. 3, the absorption spectra of the incubated YBLB media (curves *b* and *c*) showed almost identical patterns; that is, the absorption maximum at 615 nm derived from BTB disappeared in both media after the 12 h incubation period. The heat-treated (100 °C, 20 min) FVN-1 culture filtrate did not cause any spectral change when YBLB medium was used as a negative control (Fig. 3, curves *a* and *c*). Hence, these findings confirmed the crucial role of extracellular laccase in the YBLB assay.

Laccase belongs to a group of polyphenol oxidases that catalyze the oxidation of a variety of phenolic compounds, with the concomitant reduction of O_2 to water [17]. Laccases are particularly abundant in white-rot basidiomycetes, including *F. velutipes* [18]. The biological

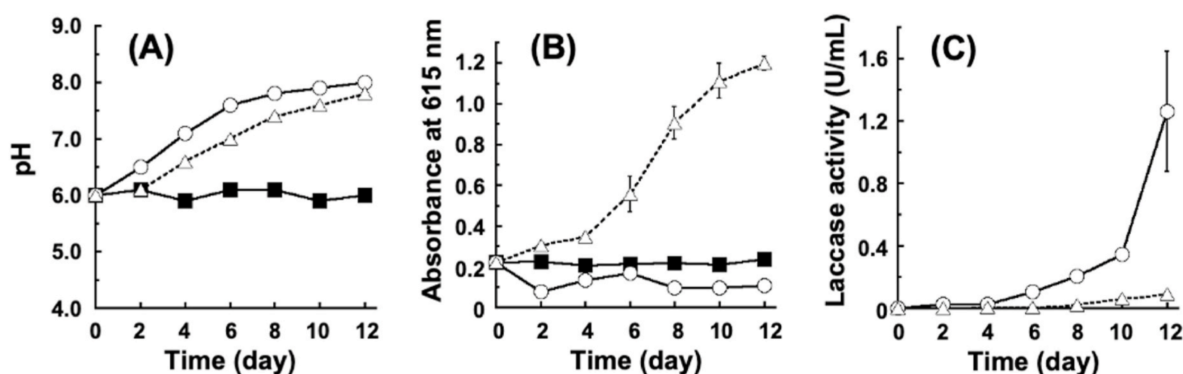

Fig. 2. Time course of the pH (A), absorbance at 615 nm (B), and laccase activity (C) of the YBLB medium inoculated with the normal (FVN-1) or degenerate (FVD-1) strain of *F. velutipes*.

The results for each medium inoculated with FVN-1 or FVD-1 are represented as open circles and triangles, respectively. The results for the uninoculated medium are represented by closed squares.

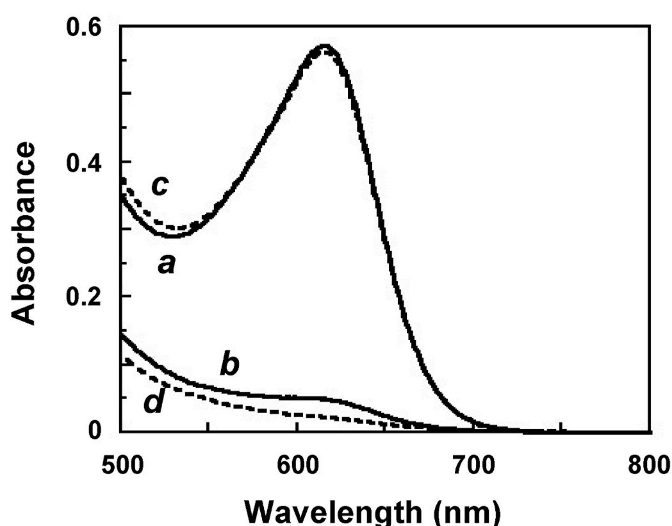

Fig. 3. Absorption spectra of the media.

The absorption spectra were obtained using a YBLB reaction mixture without enzyme solution (*a*), with culture filtrate of the normal (FVN-1) strain (*b*), with heat-denatured culture filtrate of FVN-1 (*c*), and with commercial laccase from *Trametes versicolor* (*d*). The FVN-1 filtrate from the 12-day-old culture was prepared by dialysis against 20 mM potassium phosphate buffer (pH 8.0). The YBLB reaction mixture contained YBLB medium, 4 mM potassium phosphate buffer (pH 8.0), and culture filtrate of FVN-1, heat-denatured culture filtrate of FVN-1, or commercial laccase from *T. versicolor*. The absorption spectra were obtained after incubation at 23 °C for 12 h under each condition.

functions of fungal laccases include stress defense, morphogenesis, fungal plant-pathogen/host interactions, and lignin degradation [19]. The molecular processes underlying the differentiation of mycelia into primordia are not known. Information on the biological processes of fruiting body initiation and development is also limited. In some mushrooms, laccase levels are regulated in connection with fruiting body development [20]. It has been suggested that laccase can crosslink hyphal walls into coherent aggregates during primordium formation and may continue to act on the hyphal surfaces throughout fruiting body development [21].

3.3. *Laccase production profiles of F. velutipes strains in sawdust medium*

To further analyze the difference in production levels of extracellular laccase of FVN-1 and FVD-1 strains in the YBLB assay and investigate its potential link to fruiting body development, we monitored the time course of laccase production in sawdust medium inoculated with the FVN-1 and FVD-1 strains (Supplemental Fig. S1). Laccase activities of these two strains were determined during different developmental stages. As shown in Fig. 4, laccase activity in the sawdust medium gradually increased during the initial 14 days of cultivation of the FVN-1 strain. Subsequently, a low temperature shift (from 23 °C to 15 °C) was applied, which is essential for fruiting body formation. A notable increase in laccase activity was observed in the FVN-1 culture on lowering the temperature, with the highest activity detected in the 16-old-day culture, followed by a gradual decrease in laccase activity (Fig. 4). In contrast, the laccase activity in the FVD-1 culture remained low even after the temperature shift.

Thus, a considerable difference was observed between the laccase activity profiles of the FVN-1 and FVD-1 strains determined under the same fruiting body condition, especially in the early phase of low temperature shift (i.e., 15th and 16th-day cultivation), confirming the impact of temperature change on laccase activity. Several studies have explored the potential role of laccase in the morphogenesis of some basidiomycetes, including *Pleurotus florida*, *Pleurotus ostreatus*, *Lentinula edodes*, *Hypsizygus marmoreus*, and *Ganoderma tsugae* [22–26]. However, to the best of our knowledge, this is the first report showing the relationship between fruiting body development and extracellular laccase

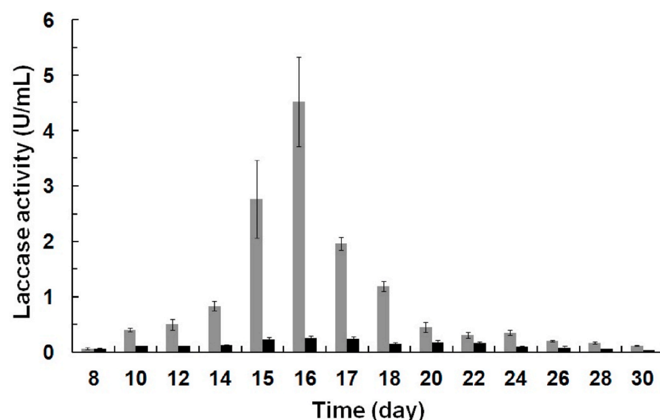

Fig. 4. Laccase activity profiles of the normal (FVN-1, gray bars) or degenerate (FVD-1, black bars) strains of *F. velutipes* during fruiting body formation in the sawdust medium.

The sawdust cultures of the FVN-1 and FVD-1 strains from each specified cultivation day were independently mixed with 4 mL of 20 mM potassium phosphate buffer (pH 8.0) and squeezed to prepare the aqueous extracts. These extracts were centrifuged twice at 14,500 rpm for 15 min at 4 °C to remove the insoluble substances, and used for laccase activity assay.

production using normal and degenerate strains of *F. velutipes*. The results obtained in this study may provide a basis for the development of an efficient cultivation method for edible mushrooms, and offer valuable new information for further studies related to the fruiting body formation in *F. velutipes* and other basidiomycete mushrooms.

Funding

This work was partially supported by JSPS KAKENHI Grant Number 20K05828.

Author contributions

Aylin Cesur: Conceptualization, Methodology, Visualization, Investigation, Writing- Original draft preparation. Ryousuke Yamamoto: Visualization, Investigation. Yasuhiko Asada: Conceptualization, Methodology, Writing-Reviewing and Editing, Supervision. Akira Watanabe: Conceptualization, Methodology, Visualization, Investigation, Writing-Original draft preparation, Writing-Reviewing and Editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that supports the findings of this study are included in this published article.

Acknowledgements

We would like to express our great appreciation to Dr. Yumi Magae for providing normal and degenerate strains of *F. velutipes*. We would like to thank Editage (www.editage.com) for English language editing.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2022.101204.

References

[1] U. Kües, Y. Liu, Fruiting body production in basidiomycetes, Appl. Microbiol. Biotechnol. 54 (2000) 141–152, https://doi.org/10.1007/s002530000396.

[2] U. Kües, M. Navarro-González, How do Agaricomycetes shape their fruiting bodies? 1. Morphological aspects of development, Fungal Biol. Rev. 29 (2015) 63–97, https://doi.org/10.1016/j.fbr.2015.05.001.

[3] Y. Sakamoto, Influences of environmental factors on fruiting body induction, development and maturation in mushroom-forming fungi, Fungal Biol. Rev. 32 (2018) 236–248, https://doi.org/10.1016/j.fbr.2018.02.003.

[4] F.M.N.A. Aida, M. Shuhaimi, M. Yazid, A.G. Maaruf, Mushroom as a potential source of prebiotics: a review, Trends Food Sci. Technol. 20 (2009) 567–575, https://doi.org/10.1016/j.tifs.2009.07.007.

[5] P. Roupas, J. Keogh, M. Noakes, C. Margetts, P. Taylor, The role of edible mushrooms in health: evaluation of the evidence, J. Funct. Foods 4 (2012) 687–709, https://doi.org/10.1016/j.jff.2012.05.003.

[6] Y. Magae, K. Akahane, K. Nakamura, S. Tsunoda, Simple colorimetric method for detecting degenerate strains of the cultivated basidiomycete *Flammulina velutipes* (Enokitake), Appl. Environ. Microbiol. 71 (2005) 6388–6389, https://doi.org/10.1128/AEM.71.10.6388-6389.2005.

[7] M. Tien, T.K. Kirk, Lignin-degrading enzyme from *Phanerochaete chrysosporium*: purification, characterization, and catalytic properties of a unique H_2O_2-requiring oxygenase, Proc. Natl. Acad. Sci. U.S.A. 81 (1984) 2280–2284, https://doi.org/10.1073/pnas.81.8.2280.

[8] H. Kofujita, Y. Asada, M. Kuwahara, Alkyl-aryl cleavage of phenolic β-*O*-4 lignin substructure model compound by Mn (II)-peroxidase isolated from *Pleurotus ostreatus*, Mokuzai Gakkaishi 37 (1991) 555–561.

[9] A. Hatakka, Lignin-modifying enzymes from selected white-rot fungi: production and role from in lignin degradation, FEMS Microbiol. Rev. 13 (1994) 125–135, https://doi.org/10.1111/j.1574-6976.1994.tb00039.x.

[10] P. Ollikka, K. Alhonmäki, V.M. Leppänen, T. Glumoff, T. Raijola, I. Suominen, Decolorization of azo, triphenyl methane, heterocyclic, and polymeric dyes by lignin peroxidase isoenzymes from *Phanerochaete chrysosporium*, Appl. Environ. Microbiol. 59 (1993) 4010–4016, https://doi.org/10.1128/aem.59.12.4010-4016.1993.

[11] P. Verma, D. Madamwar, Decolorization of synthetic textile dyes by lignin peroxidase of *Phanerochaete chrysosporium*, Folia Microbiol. 47 (2002) 283–286, https://doi.org/10.1007/BF02817653.

[12] P.P. Champagne, J.A. Ramsay, Contribution of manganese peroxidase and laccase to dye decoloration by *Trametes versicolor*, Appl. Microbiol. Biotechnol. 69 (2005) 276–285, https://doi.org/10.1007/s00253-005-1964-8.

[13] T. Nitheranont, A. Watanabe, Y. Asada, Extracellular laccase produced by an edible basidiomycetous mushroom, *Grifola frondosa*: purification and characterization, Biosci. Biotechnol. Biochem. 75 (2011) 538–543, https://doi.org/10.1271/bbb.100790.

[14] K. Otsuka Saito, R. Ikeda, K. Endo, Y. Tsujino, M. Takagi, E. Tamiya, Isolation of a novel alkaline-induced laccase from *Flammulina velutipes* and its application for hair coloring, J. Biosci. Bioeng. 113 (2012) 575–579, https://doi.org/10.1016/j.jbiosc.2012.01.001.

[15] J. Yao, R. Jia, L. Zheng, B. Wang, Rapid decolorization of azo dyes by crude manganese peroxidase from *Schizophyllum* sp. F17 in solid-state fermentation, Biotechnol. Bioproc. Eng. 18 (2013) 868–877, https://doi.org/10.1007/s12257-013-0357-6.

[16] Z. Duan, R. Shen, B. Liu, M. Yao, R. Jia, Comprehensive investigation of a dye-decolorizing peroxidase and a manganese peroxidase from *Irpex lacteus* F17, a lignin-degrading basidiomycete, AMB Express 8 (2018) 119, https://doi.org/10.1186/s13568-018-0648-6.

[17] C.F. Thurston, The structure and function of fungal laccases, Microbiology 140 (1994) 19–26, https://doi.org/10.1099/13500872-140-1-19.

[18] K. Brijwani, A. Rigdon, P.V. Vadlani, Fungal laccases: production, function, and applications in food processing, Enzym. Res. 2010 (2010) 149748, https://doi.org/10.4061/2010/149748.

[19] D.M. Mate, M. Alcalde, Laccase: a multi-purpose biocatalyst at the forefront of biotechnology, Microb. Biotechnol. 10 (2017) 1457–1467, https://doi.org/10.1111/1751-7915.12422.

[20] D. Singh Arora, R. Kumar Sharma, Ligninolytic fungal laccases and their biotechnological applications, Appl. Biochem. Biotechnol. 160 (2010) 1760–1788, https://doi.org/10.1007/s12010-009-8676-y.

[21] P. Giardina, V. Faraco, C. Pezzella, A. Piscitelli, S. Vanhulle, G. Sannia, Laccases: a never-ending story, Cell. Mol. Life Sci. 67 (2010) 369–385, https://doi.org/10.1007/s00018-009-0169-1.

[22] N. Das, S. Sengupta, M. Mukherjee, Importance of laccase in vegetative growth of *Pleurotus florida*, Appl. Environ. Microbiol. 63 (1997) 4120–4122, https://doi.org/10.1128/aem.63.10.4120-4122.1997.

[23] X. Jiao, G. Li, Y. Wang, F. Nie, X. Cheng, M. Abdullah, Y. Lin, Y. Cai, Systematic analysis of the *Pleurotus ostreatus* laccase gene (*PoLac*) family and functional characterization of *PoLac2* involved in the degradation of cotton-straw lignin, Molecules 23 (2018) 880, https://doi.org/10.3390/molecules23040880.

[24] S. Ohga, D.J. Royse, Transcriptional regulation of laccase and cellulase genes during growth and fruiting of *Lentinula edodes* on supplemented sawdust, FEMS Microbiol. Lett. 201 (2001) 111–115, https://doi.org/10.1111/j.1574-6968.2001.tb10741.x.

[25] J. Zhang, H. Chen, M. Chen, A. Ren, J. Huang, H. Wang, M. Zhao, Z. Feng, Cloning and functional analysis of a laccase gene during fruiting body formation in *Hypsizygus marmoreus*, Microbiol. Res. 179 (2015) 54–63, https://doi.org/10.1016/j.micres.2015.06.005.

[26] W. Jin, J. Li, H. Feng, S. You, L. Zhang, J. Norvienyeku, K. Hu, S. Sun, Z. Wang, Importance of a laccase gene (*Lcc1*) in the development of *Ganoderma tsugae*, Int. J. Mol. Sci. 19 (2018) 471, https://doi.org/10.3390/ijms19020471.