Utilization of Agroindustrial Wastes for the Production of Laccase by *Pleurotus eryngii* Han 1787 and *Lentinus edodes* Han1788

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Laccase activity secreted by *Pleurotus eryngii* Han 1787 and *Lentinus edodes* Han 1788 grown on six types of agroindustrial wastes was investigated. Maximum laccase activity of *P. eryngii* Han 1787 on *Ulmus pumila*, *Juniperus formosana*, *Pinus tabuliformis*, cottonseed shell, corncob, and leaf of corncob was nearly 5.77-fold, 2.37-fold, 2.78-fold, 2.81-fold, 11.53-fold, and 6.73-fold higher than that of *L. edodes* Han1788 on corresponding agroindustrial wastes. In general, the capacity of secreting laccase of *P. eryngii* Han 1787 was superior to that of *L. edodes* Han 1788. Furthermore, laccase activity of *P. eryngii* Han 1787 on the leaf of corncob, the corncob, *Ulmus pumila*, and *Juniperus formosana* was relatively stable during the whole fermentation process. Different fungi showed different preferences in different agroindustrial wastes to secret laccase on whole fermentation stage. The presence of leaf of corncob was useful for improving laccase activity of *P. eryngii* Han 1787, while *L. edodes* Han 1788 was more preferred to produce laccase along with the presence of *Juniperus formosana*. These results were preliminary conducive in laying the foundation for increasing industrial laccase-producing strains and producing low-cost laccase.

DOI: 10.15376/biores.18.1.570-583

Keywords: White-rot fungi; *Pleurotus eryngii*; *Lentinus edodes*; *Laccase activity*; *Preference of agroindustrial wastes*

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INTRODUCTION

Lignocellulosic biomass, deemed as one of the most promising, profuse, and renewable natural resources, is mainly composed of cellulose (30 to 50% of the dry matter weight), hemicellulose (20 to 40% of the dry matter weight), and lignin (15 to 25% of the dry matter weight), in addition to a small amount of structural proteins, lipids, and ash (Mosier *et al*. 2005; Unuofin *et al*. 2019a; Han *et al*. 2021b). Large quantities of lignocellulosic biomass are abandoned by various industries, such as agricultural, forestry, and paper pulp (Birhanli and Yesilada 2013). These lignocellulosic biomasses, treated as agroindustrial wastes, are carelessly discarded and burned, which then leads to serious environmental pollution. The chemical properties of agroindustrial wastes allow them to be reused constructively. For example, certain waste products can be used as substrates for crucial complex fermentation in biotechnological applications (Howard *et al*. 2003; Unuofin *et al*. 2019b). However, while the hemicellulose and cellulose components can be

degraded by numerous microorganisms, the lignin—the most resistant component of agroindustrial wastes to microbial degradation—can only be efficiently converted by a few organisms (Sanchez 2009). Lignin acts as a barrier to protect cellulose and hemicellulose from enzymatic attack; thus the degradation of lignocellulose requires good breaking of the lignin barrier (Choi *et al*. 2019). Among the numerous microorganisms, white rot fungi are considered to be effective in breaking the lignin barrier due to their ability to secrete extracellular lignocellulolytic enzymes such as polyphenol oxidase and peroxidase (Dinis *et al*. 2009; Birhanli and Yesilada 2010; Muthuvelu *et al*. 2020). Laccases are classified as polyphenol oxidases and are typically involved in lignin degradation.

Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2), belongs to a family of multicopper-containing enzymes, and is an ancient and important enzyme due to the low specificity of its substrate (Baldrian 2006; Agrawal *et al*. 2018; An *et al*. 2020; Habimana *et al*. 2021). Laccase is produced by higher plants, bacteria, insects, and fungi (Yang *et al*. 2017; Srinivasan *et al*. 2019; Nuskern *et al*. 2021) and has huge application potential in different biotechnological, industrial, and environmental fields, such as biopulping, bioremediation, biodegradation, biosensors, nanoscience, and beverage and beer industry (Bertrand *et al.* 2017; Kudanga *et al*. 2017; Mate and Alcalde 2017; Jaya Mary *et al*. 2018; Su *et al*. 2018; Yashas *et al*. 2018; Deska and Konczak 2019; Zerva *et al*. 2019; Liu *et al*. 2020b). However, the extensive application of laccase requires the support of many high activity and low-cost laccase sources (Couto and Toca-Herrera 2007). Therefore, it is important for industrial applications to select for suitable fermentation methods with cheap and widespread carbon/nitrogen sources; these sources ideally from efficient laccase-producing microorganisms to effectively produce laccase (Birhanli and Yesilada 2013; Mate and Alcalde 2017; Rodrigues *et al*. 2019; Habimana *et al*. 2021).

Agroindustrial wastes, such as sugarcane bagasse and oil palm frond petiole, have been commonly used as substrates for fungal fermentation to produce laccase (Ikubar *et al*. 2018). Amongst the numerous processes used for producing enzyme, using agroindustrial wastes to perform the solid-state fermentation (SSF) is an attractive and cost-effective option (Couto and Sanroman 2005). Compared to the submerged fermentation (SF), the main advantages of SSF are low water requirements, no dilution of enzymes, and relatively simple control technology. It is particularly important to use agroindustrial wastes for solid-state fermentation to provide conditions for the attachment of fungal mycelia, which is more similar to their natural environment of fungi. The production of ligninolytic enzymes, such as laccase, is affected by many factors including fungal species, medium components, and types of substrates (Liu *et al*. 2009; Hu *et al*. 2014; Martin *et al*. 2021). Thus, it is very important to evaluate enzyme production of new isolated fungal strains when investigating new substrates to identify suitable types of agroindustrial wastes in the medium. A previous study investigated the effects of mixed agro-residues (corn crop waste) on lignin-degrading enzyme activities, growth, and quality of *Lentinula edodes* (Xu *et al*. 2020). However, studies on laccase activity induced by a single lignocellulosic material are lacking.

Therefore, the main aim of the present study was to investigate the feasibility of using *Ulmus pumila*, *Pinus tabuliformis*, cottonseed shell, corncob, leaf of corncob, and *Juniperus formosana*, as natural, low-cost substrates for producing laccase by *Pleurotus eryngii* Han 1787 and *Lentinus edodes* Han 1788 *via* conventional solid-state fermentation in short time periods. Some of these agroindustrial wastes (*Ulmus pumila*, leaf of corncob, and *Juniperus formosana*) were first used for producing laccase by *Pleurotus eryngii* and *Lentinus edodes*. The results are preliminary conducive to lay a foundation for increasing industrial laccase-producing strains and producing low-cost laccase.

EXPERIMENTAL

Materials

Microorganisms

Fruiting bodies of two fungi were purchased in supermarkets from Langfang city, Hebei province, China. Under aseptic conditions, a few fresh fruiting bodies were scraped with forceps and inoculated on a PDA medium (peeled potatoes 100 g, glucose 10 g, agar 10 g, deionized water 1 L) petri dish. After 5 days, small pieces of culture medium with mycelium were extracted with an inoculating shovel and transferred to a new PDA medium petri dish. The strain numbers of two fungi were Han 1787 and Han 1788. Two strains were preserved on Malt Extract Agar (MEA) medium (glucose 10 g, malt extract 20 g, KH_2PO_4 3 g, agar 20 g, deionized water 1 L) and the culture medium composition, according to An *et al*. (2021b). Two strains were stored in the College of Life Science, Langfang Normal University (Langfang, China).

Agroindustrial wastes

Cottonseed shell, corncob, trunk of *Ulmus pumila*, trunk of *Pinus tabuliformis*, and leaf of corncob were kindly provided by farmers of Chengde city, Hebei province. The trunk of *Juniperus formosana* was collected from Langfang city, Hebei province. All agroindustrial wastes were air-dried and ground into small particles with the size between 40- and 80-mesh by a micro plant grinding machine FZ-102 (Tianjin Taiste Instrument Co., Ltd.).

Methods

Microbial culture

Han 1787 and Han 1788 were reactivated on PDA medium at 26 ℃ for 9 days. Five round pieces with a diameter of 5 mm were transferred to 100 mL PDA liquid medium at 26 ℃ with a speed of 150 rpm to perform the stable oscillation culture. After 7 days, mycelium pellets were homogenized by a hand-held blender to prepare the subsequent inoculum.

Solid-state fermentation and acquisition of crude enzyme solution

Two grams of dry agroindustrial wastes moistened with 10 mL liquid ($MgSO_4 \cdot 7H_2O$ 0.5 g, K_2HPO_4 1 g, KH_2PO_4 0.46 g, $CaCl_2$ 0.2 g, deionized water 1 L) were added to a 250-mL Erlenmeyer flask. After sterilization, 3 mL homogenized inoculum was added to all flasks. Then, all flasks were transferred to an incubator at constant temperature (26 °C) to perform the process of solid-state fermentation. The experiment was performed in triplicate. To obtain crude enzyme, the flasks were added into 100 mL acetate-sodium acetate buffer (50 mM) at pH 5.5 and agitated on a shaker at 10 °C with a speed of 120 rpm for 4 h, according to An *et al*. (2021a). Then, the liquor was filtered through filter paper to remove the agroindustrial wastes and centrifuged at 4 °C with a speed of 12,000 rpm for 20 min. The supernatant was used to detect laccase activity.

Measurement of laccase activity

Laccase activity was determined by the method of 2,2'-azinobis-[3-ethyltiazoline-6-sulfonate] (ABTS) *via* measurement of the optical density (OD) at 415 nm (Bourbonnais and Paice 1990). The ABTS method is detailed by Han *et al*. (2021b). One activity unit was defined as the amount of enzyme required per minute for the oxidation of 1 μmol

ABTS (ε_{415} nm = 3.16×10^4 M^{-1} cm^{-1}).

Data analysis

The effects of agroindustrial wastes and strains on laccase activity were examined through two-way analysis of variance according to Han *et al*. (2021b) by the SPSS 22.0 program (PROC GLM, Armonk, NY, USA). All figures were generated by Origin 2016 (OriginLab Corporation, Northampton, MA, USA).

Identification of the fungi

Mycelia of Han 1787 and Han 1788 used for DNA extraction were obtained from a petri dish containing CYM medium (glucose 20 g, peptone 2 g, yeast extract 2 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $K_2HPO_4 \cdot 3H_2O$ 1 g, KH_2PO_4 0.46 g, agar 15 g, deionized water 1 L) with fungi grown for 7 days. An appropriate amount of mycelium used for extracting DNA after grinding was scraped from the surface of the medium with a sterile surgical blade and transferred to an EP tube.

The extraction method was appropriately modified according to the instructions of the genomic DNA rapid extraction Kit-DN14 (Aidlab Biotechnologies Co., Ltd., Beijing, China) and the method of Han *et al*. (2016, 2021a). Universal primers ITS5 and ITS4 were used for PCR amplification to obtain ITS sequences of these two strains.

The PCR amplification procedure and system were according to Han *et al*. (2016, 2021a), and the products were sent to Beijing Genomics Institute (Beijing, China) for sequencing. Blast comparison of ITS sequences was performed on the NCBI website to identify the strains.

RESULTS AND DISCUSSION

Identification of Fungal Species

Previous studies have also used ITS sequences for fungal species identification (Han *et al*. 2016, 2021b). The identification result of Han 1787 and Han 1788 was *Pleurotus eryngii* and *Lentinus edodes*, and corresponding Genbank No. was ON911812 and ON911813.

Results of Statistical Analysis

The effect of fungal species and agroindustrial wastes on laccase activity was significant ($P < 0.001$) during the whole stage of solid-state fermentation. Meanwhile, the interactions of fungal species and agroindustrial wastes on laccase activity were also significant ($P < 0.001$) (Table 1).

Table 1. Two-way ANOVA to Exam the Effects of Strains, Agroindustrial Wastes, and the Interactions between Strains and Agroindustrial Wastes on Laccase Activities

Incubation Period (d)	Strains	Agroindustrial Wastes	Strains × Agroindustrial Wastes
1	1964.424***	1153.231***	1100.500***
2	1031.458***	350.978***	349.706***
3	2656.214***	251.165***	250.707***
4	4790.844***	299.303***	293.178***
5	3052.528***	197.158***	195.713***
6	15124.815***	1079.281***	1039.806***
7	10785.423***	598.134***	605.022***
8	2773.903***	211.058***	265.831***
9	5542.250***	410.407***	365.060***
10	5276.387***	336.236***	282.855***
11	5111.133***	435.240***	283.299***
12	1531.056***	118.079***	87.637***
13	2662.578***	168.700***	171.318***
14	14588.637***	762.926***	1197.787***
15	2462.918***	296.317***	287.247***
Note: df = 1, 5, 5; ***P < 0.001; The values were the F-value of Two-way ANOVA.			

Species-dependent Laccase Production

The white-rot basidiomycetes have the ability to produce hydrolytic enzymes that are involved in hydrolyzing cellulose and hemicellulose, as well as ligninolytic enzymes that are involved in degrading lignin during whole fermentation of lignocellulose (Elissetche *et al*. 2007; Birhanli and Yesilada 2013; Thamvithayakorn *et al*. 2019). Similarly, previous studies had indicated that *Lentinula edodes* and *Pleurotus eryngii* have the capacity to produce laccase (Yan *et al*. 2019; Liu *et al*. 2020a; Zhang *et al*. 2020, 2021). Furthermore, many studies have shown that different species of fungi have different abilities in producing ligninase (Guo *et al*. 2017; Huang *et al*. 2019; An *et al*. 2021a). Screening and obtaining high-yield laccase strains is of great significance for industrial application of laccase. In this study, the activity of laccase of *P. eryngii* Han 1787 and *L. edodes* Han 1788 fermented on six readily available agroindustrial wastes was evaluated for the first time.

Due to the laccase activity from *Pleurotus eryngii* Han 1787 and *Lentinus edodes* Han1788 fermented on *Ulmus pumila*, *Juniperus formosana*, *Pinus tabuliformis*, cottonseed shell, corncob, and leaf of corncob for the 1st day (Figs. 1, 2), it can be seen that laccase is produced faster by *P. eryngii* Han 1787 than by *L. edodes* Han 1788. Results also indicated that the laccase-producing ability of different fungi was very different. Similarly, An *et al*. (2021a) evaluated the capacity of secreting laccase of *P. ostreatus* CY 568 and *Ganoderma lingzhi* Han 500 and found that the capacity of secreting laccase from *P. ostreatus* CY 568 was stronger than that from *G. lingzhi* Han 500.

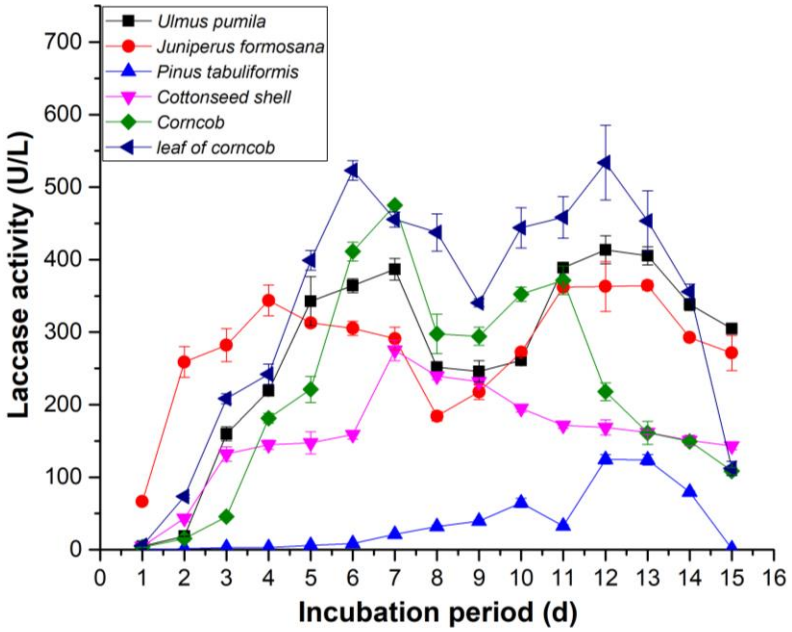

Fig. 1. Laccase activity from *Pleurotus eryngii* Han 1787 fermented on *Ulmus pumila*, *Juniperus formosana*, *Pinus tabuliformis*, cottonseed shell, corncob, and leaf of corncob

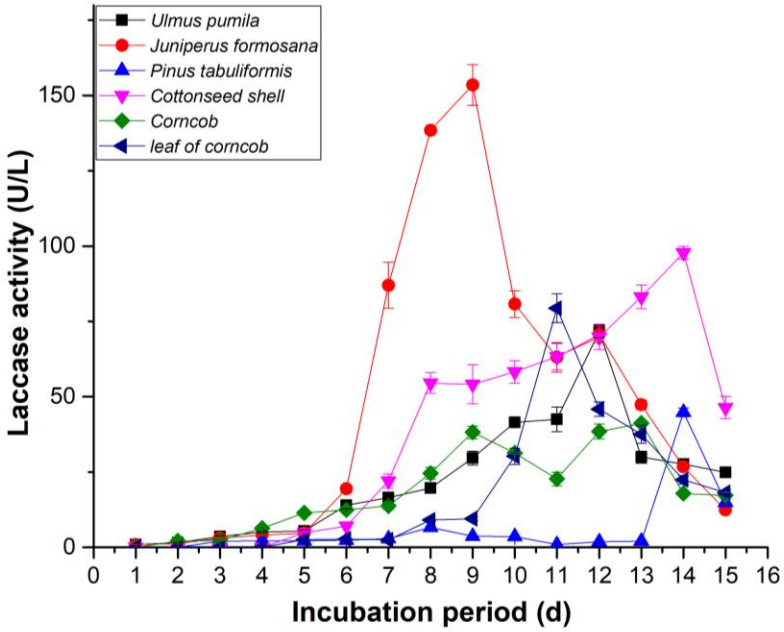

Fig. 2. Laccase activity from *Lentinus edodes* Han 1788 fermented on *Ulmus pumila*, *Juniperus formosana*, *Pinus tabuliformis*, cottonseed shell, corncob, and leaf of corncob

According to the data shown in Table 2, maximum laccase activity of *P. eryngii* Han 1787 on *Ulmus pumila*, *Juniperus formosana*, *Pinus tabuliformis*, cottonseed shell, corncob, and leaf of corncob was nearly 5.77-fold, 2.37-fold, 2.78-fold, 2.81-fold, 11.53-fold, and 6.73-fold higher than that of *L. edodes* Han1788 on corresponding agroindustrial wastes, relatively. In general, the capacity of secreting laccase of *P. eryngii* Han 1787 was superior to that of *L. edodes* Han 1788. Previous studies indicated that laccase activity was

affected by fungal species or strains (Elisashvili *et al.* 2008b; An *et al*. 2016; Han *et al*. 2017; Huang *et al.* 2019; An *et al*. 2021b; Han *et al.* 2021b). Maximum laccase activities obtained from solid-state fermentation cultures of *Trametes trogii* and *T. versicolor* were 384 U/L (day 10) and 68 U/L (day 8), respectively, when fermented on *Corylus maxima* (Birhanli and Yesilada 2013). Stajic *et al*. (2006) found that laccase production of *P. eryngii*, strain No. 616, fermented on grapevine sawdust was 42 ± 1 and 9.3 ± 0.2 U/L, after 5 and 7 days, respectively, which was higher than the results of Martinez *et al*. (1994) and Munoz *et al*. (1997). However, maximum laccase activity of *P. eryngii* Han 1787 in the present study was 533.84 ± 51.48 U/L fermented on leaf of corncob, which is higher than the results of previous studies. Laccase activity of *L. edodes* IBB 123, *L. edodes* IBB 363, and *L. edodes* IBB 369 fermented on tree leaves and wheat straw was 57 ± 4.7 U/flask, 52 ± 4.9 U/flask, 7 ± 0.7 U/flask and 20 ± 1.5 U/flask, 55 ± 5.1 U/flask, 38 ± 4.0 U/flask (Elisashvili *et al*. 2008b). In this study, maximum laccase activity of *L. edodes* Han 1788 on *Juniperus formosana* was 153.51 ± 6.74 U/L. Overall, the activity of laccase produced by *P. eryngii* Han 1787 and *L. edodes* Han 1788 in this study was higher than that from the same species in previous studies.

Table 2. Maximum Laccase Activity Value and Occurrence Time of *Pleurotus eryngii* Han 1787 and *Lentinus edodes* Han 1788 Fermented on Different Agroindustrial Wastes

Maximum Laccase Activity (U/L)	Agroindustrial Wastes	Strains	Time (Day)
413.60 ± 19.30	*Ulmus pumila*	*Pleurotus eryngii* Han 1787	12th
364.38 ± 6.65	*Juniperus formosana*	*Pleurotus eryngii* Han 1787	13th
124.67 ± 6.64	*Pinus tabuliformis*	*Pleurotus eryngii* Han 1787	12th
274.96 ± 14.38	Cottonseed shell	*Pleurotus eryngii* Han 1787	7th
474.88 ± 0.17	Corncob	*Pleurotus eryngii* Han 1787	7th
533.84 ± 51.48	leaf of corncob	*Pleurotus eryngii* Han 1787	12th
71.73 ± 2.17	*Ulmus pumila*	*Lentinus edodes* Han 1788	12th
153.51 ± 6.74	*Juniperus formosana*	*Lentinus edodes* Han 1788	9th
44.81 ± 1.52	*Pinus tabuliformis*	*Lentinus edodes* Han 1788	14th
97.85 ± 2.14	Cottonseed shell	*Lentinus edodes* Han 1788	14th
41.19 ± 0.97	Corncob	*Lentinus edodes* Han 1788	13th
79.37 ± 4.79	leaf of corncob	*Lentinus edodes* Han 1788	11th
Data are presented as mean value ± standard deviation for biological triplicates and are expressed as U/L.			

Effects of Agroindustrial Wastes on Laccase Activity

Solid material used to culture fungi can be inert or non-inert material. The inert material acts only as an attachment site for the fungus, while the non-inert material acts not only as an attachment site, but also provides some nutrients for the fungus. Therefore, noninert materials are called support substrate (Couto and Sanroman 2005; Birhanli and Yesilada 2013; Leite *et al*. 2019; Lizardi-Jimenez *et al*. 2019). Because agroindustrial wastes contain three major components (lignin, cellulose, and hemicellulose) that are rich in sugar, the wastes can act as an attachment place for mycelium and also provide nutrition for mycelium growth. Thus, one of the most appropriate approaches for ensuring the efficient production of laccase is the utilization of agroindustrial wastes (Birhanli and Yesilada 2013; Gupta and Jana 2019). The agroindustrial wastes commonly used in previous studies were poplar, *Juglans regia*, *Triticum sativum*, and sugarcane bagasse (Gaikwad and Meshram 2019; Gujjala *et al*. 2019; Nawaz *et al*. 2019; Wang *et al*. 2019; Atilano-Camino *et al*. 2020; Malhotra and Suman 2021). Thus, evaluation of more low-cost agroindustrial wastes is essential for low-cost laccase production.

Maximum laccase activity of *Pleurotus eryngii* Han 1787 on *Ulmus pumila*, *Juniperus formosana*, *Pinus tabuliformis*, cottonseed shell, corncob, and leaf of corncob was 413.60 ± 19.30 U/L, 364.38 ± 6.65 U/L, 124.67 ± 6.64 U/L, 274.96 ± 14.38 U/L, 474.88 ± 0.17 U/L, and 533.84 ± 51.48 U/L (Fig. 1, Table 2). Thus, the existence of leaf of corncob was conducive for *P. eryngii* Han 1787 to secret laccase. Furthermore, corncob was also contributed to increase laccase activity compared to *Ulmus pumila*, *Juniperus formosana*, *Pinus tabuliformis*, and cottonseed shell. The possible reason is that the cellulose content of leaf of corncob and corncob was relatively higher than other tested agroindustrial wastes and beneficial to the accumulation of mycelial biomass of fungi. Meanwhile, maximum laccase activity of *Lentinus edodes* Han 1788 on *Ulmus pumila*, *Juniperus formosana*, *Pinus tabuliformis*, cottonseed shell, corncob, and leaf of corncob was 71.73 ± 2.17 U/L, 153.51 ± 6.74 U/L, 44.81 ± 1.52 U/L, 97.85 ± 2.14 U/L, 41.19 ± 0.97 U/L, and 79.37 ± 4.79 U/L (Fig. 2, Table 2). Obviously, the presence of *Juniperus formosana* was contributed to *L. edodes* Han 1788 for enhancing laccase activity in a short time. Maximum laccase activity of *Lentinus edodes* strain 122 fermented on WS (*Triticum aestivum*), RG (mixture of *Typha angustifolia*, *Carex pseudocyperus* and *Phragmites australis*) and BS (*Phaseolus coccineus*) was about 540 U/g (3.5 weeks), 260 U/g (3.5 weeks), and 390 U/g (5 weeks) (Philippoussis *et al*. 2011). Obviously, although the maximum laccase activity of *L. edodes* Han 1788 used in this study was only 153.51 ± 6.74 U/L detected on *Juniperus formosana*, lower than *L. edodes* strain 122 detected on *Triticum aestivum* (540 U/g) in the previous study, the occurrence time of maximum laccase activity of *L. edodes* Han 1788 (at 9th day) was much earlier than *L. edodes* strain 122 (at 3.5 weeks). Maximum laccase activities obtained from solid-state fermentation cultures of *Trametes versicolor* was 107 U/L (day 8), 62 U/L (day 10), 68 U/L (day 8), 107 U/L (day 8), 387 U/L (day 10), and 215 U/L (day 10) when fermented on *Helianthus annuus*, *Juglans regia*, *Corylus maxima*, *Armeniaca vulgaris*, *Zea mays*, and *Triticum sativum* (Birhanli and Yesilada 2013). Laccase of *Funalia trogii* IBB 146 on tree leaves, wheat straw, apple peels, and banana peels was 458 ± 54 U/L, 760 ± 70 U/L, 211 ± 24 U/L, and 988 ± 74 U/L (Elisashvili *et al*. 2008a). Thus, results show a significant difference in laccase activity of fungal strains fermented on different agriculture and forestry residues. Similarly, this study also found that different fungi have different preferences for agroindustrial wastes.

CONCLUSIONS

1. Overall, the capacity of secreting laccase of *Pleurotus eryngii* Han 1787 on all tested agroindustrial wastes was superior to that of *Lentinus edodes* Han 1788 during the whole fermentation process.
2. The effect of six types of agroindustrial wastes on laccase activity of *P. eryngii* Han 1787 was: leaf of corncob > corncob > *Ulmus pumila* > *Juniperus formosana* > cottonseed shell > *Pinus tabuliformis*. In other words, the presence of leaf of corncob was useful for improving laccase activity of *P. eryngii* Han 1787.
3. The effect of six types of agroindustrial wastes on laccase activity of *L. edodes* Han 1788 was: *Juniperus formosana* > cottonseed shell > leaf of corncob > *Ulmus pumila* > *Pinus tabuliformis* > corncob. In other words, the presence of *Juniperus formosana* was useful for improving laccase activity of *L. edodes* Han 1788.
4. Maximum laccase activity of *P. eryngii* Han 1787 on leaf of corncob was 533.84 ± 51.48 U/L, nearly 1.29-fold, 1.47-fold, 4.28-fold, 1.94-fold, and 1.12-fold higher than that on *Ulmus pumila*, *Juniperus formosana*, *Pinus tabuliformis*, cottonseed shell, and corncob, and nearly 7.44-fold, 3.48-fold, 11.91-fold, 5.46-fold, 12.96-fold, and 6.73-fold higher than *L. edodes* Han 1788 on *Ulmus pumila*, *Juniperus formosana*, *Pinus tabuliformis*, cottonseed shell, corncob, and leaf of corncob, respectively.

ACKNOWLEDGMENTS

This research was supported by the National Natural Science Foundation of China (31900009), the Science and Technology Project of Hebei Education Department (BJK2022052), the Fundamental Research Funds for the Universities in Hebei Province (JYQ202201 & JYQ201901), and the Top-notch Youth Project of Langfang City, China.

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Article submitted: July 7, 2022; Peer review completed: September 24; Revised version received and accepted: November 8, 2022; Published: November 17, 2022.
DOI: 10.15376/biores.18.1.570-583