Effect of Alkali Lignin on Laccase Activity and Mycelial Biomass of *Flammulina velutipes*

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The effect of alkali lignin on laccase activity and mycelial biomass of *Flammulina velutipes* was investigated at different fermentation temperatures. The secretion of laccase by *F. velutipes* has certain specificity. Under most fermentation medium conditions, no laccase activity of *F. velutipes* CCMSSC 00103 was detected, but laccase activity of *F. velutipes* FL 19 could be detected. Among the three strains of *F. velutipes*, the laccase secretion capacity of *F. velutipes* FL 19 was clearly higher than that of *F. velutipes* CCMSSC 05331 and *F. velutipes* CCMSSC 00103. Maximum laccase activity with the value of 73.42 ± 4.74 U/L was secreted by *F. velutipes* FL 19 in fermentation medium with alkali lignin, KH_2PO_4, Vitamin B1, and glucose, and appeared on the 2nd day. The presence of alkali lignin was useful for improving laccase activity secreted by *F. velutipes.* The effect of alkali lignin on improving laccase activity exceeded that of *Populus beijingensis*. *F. velutipes* was more suitable for secreting laccase at 26 °C because the maximum laccase activity at 26 °C (73.42 ± 4.74 U/L) was higher than that measured at 33 °C (70.71 ± 6.89 U/L). The results can be useful for selecting suitable medium and temperature of *F. velutipes* strains to produce low-cost laccase.

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INTRODUCTION

Lignin, the most abundant and natural aromatic substance in nature, is a polymer composed of phenylpropyl units connected by various non-hydrolyzed C−C and C−O−C bonds (Yang *et al*. 2012; Yang *et al*. 2021). Because of its extremely complex structure, which is mainly reflected in the irregular structure of the matrix and the high branching of the polymer network, lignin has a strong ability to resist microbial decomposition (Muaaz-Us-Salam *et al*. 2020). Microbial depolymerization of lignin, which is one of the important mechanisms of lignin depolymerization, has the advantages of low energy consumption, no chemical additives, and environmental friendliness (Niu *et al*. 2021). A breakthrough in the field of lignin biodegradation was investigated in 1983 when Tien and Kirk (1983) described fungal lignin enzymes and their peroxide requirements. This finding led to the biodegradation of lignin by fungi being widely studied worldwide (Pointing *et al*. 2001;

An *et al*. 2023). Thus, fungi secreting related enzymes involved in the efficiently degradation of lignin, including manganese peroxidase (MnP), lignin peroxidase (Lip), and laccase (Lac), have been extensively studied (Wong 2009; Barapatre and Jha 2017; Yang *et al*. 2021). Manganese peroxidase is a glycosylated hemoglobin that converts lignin phenolic compounds to phenoxy radicals, while lignin peroxidase is a glycoprotein that oxidizes nonphenolic and phenolic compounds. Laccase, belonging to a family of multi-copper oxidase, can destroy the internal stability of aromatic rings by oxidizing the phenolic hydroxyl of lignin (Xu *et al*. 2018; Weng *et al*. 2021). In addition, laccase has been widely studied globally due to its wide and remarkable range of natural substrates (An *et al*. 2021a, 2021b).

Laccase (EC 1.10.3.2), also commonly known as p-diphenol oxidase, is a type of oxidase that catalyzes the reduction of O_2 to H_2O (Singh *et al*. 2011; Wang *et al*. 2019). Laccase was first described by Yoshida (1883) and discovered in Chinese or Japanese lacquer tree's latex (Strong and Claus 2011). Laccase has been found in higher plants, bacteria, insects, actinomycetes, and fungi of deuteromycetes, ascomycetes, and basidiomycetes, which indicates that laccase is involved in various physiological processes (Chen *et al*. 2013; Chauhan *et al*. 2017; Geng *et al*. 2018; Khatami *et al*. 2022). At present, the biotechnological applications of laccases have been investigated for more than 30 years because they are widely used in a wide scope of disciplines. Laccase has been shown to be useful in the paper and bio-pulping industry, cosmetics and pharmaceuticals industry, sewage treatment industry, chemical industry, textile industry, energy industry, bioremediation industry, food and feed industry, and many others (Minussi *et al*. 2002; Senthivelan *et al*. 2016; Yashas *et al*. 2018; Navas *et al*. 2019; Singh and Arya 2019; Li *et al*. 2022; Zhang *et al*. 2022; Dong *et al*. 2023). However, the high production cost, low activity, and low stability of laccase prevent its widespread use in industry (Couto and Toca-Herrera 2007; Goncalves *et al*. 2015; Su *et al*. 2018).

There are many factors affecting the activity of fungal laccase, such as pH, temperature, the type of fungus, lignocellulosic material, compounds, the type and concentration of metal ions, *etc.* (Hu *et al*. 2014; Bettin *et al*. 2019; Rodriguez *et al*. 2019; Sun *et al*. 2021; Tiwari *et al*. 2023; Vandelook *et al*. 2024). Thus, many researchers are working to develop new microorganisms that are capable of producing laccase (Yang *et al*. 2015; Hadibarata *et al*. 2018; Rao *et al*. 2019; Han *et al*. 2021a, 2021b; Oztat *et al*. 2024). Meanwhile, the ability of fungal mixtures to produce laccase has also been investigated (Verma and Madamwar 2002; Vibha and Negi 2018; Rodriguez *et al*. 2019; Zhang *et al*. 2020). Of course, there are also scholars committed to exploring the types of lignocellulosic materials or methods involving the mixing of lignocellulosic materials to analyze the effects on the production of laccase by fungi (Xu *et al*. 2020; Han *et al*. 2021b). In addition, there have been some studies analyzing the effects of metal ions or aromatic compounds on the activity of fungal laccase (Wang *et al*. 2011; Yang *et al*. 2016; Zhuo *et al*. 2017; Li *et al*. 2022). *Flammulina velutipes*, as a model white-rot fungus, had been shown to produce laccase in previous studies (Janusz *et al*. 2015; An *et al*. 2016; Cesur *et al*. 2022a,b). These studies mainly focused on the effects of metal ions, lignocellulose materials, and aromatic compounds on laccase activity secreted by *F. velutipes* strains. However, there has been a need for studies on the effect of alkali lignin on laccase activity secreted by *F. velutipes*. Under these circumstances, this study intended to analyze the effect of alkali lignin on laccase activity and mycelial biomass of *F. velutipes*. Meanwhile, the differences in laccase activity and mycelial biomass from different *F. velutipes* strains were investigated. The results will contribute to produce low-cost laccase.

EXPERIMENTAL

Materials

Microorganisms

Flammulina velutipes CCMSSC 05331, *F. velutipes* FL 19, and *F. velutipes* CCMSSC 00103 were kindly provided by Institute of Microbiology, Beijing Forestry University. All fungal species were stored on malt extract agar (MEA) medium (glucose 10 g/L, malt extract 20 g/L, KH_2PO_4 3 g/L, and agar 20 g/L).

Chemicals

2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and alkali lignin were purchased from Sigma-Aldrich (Sigma Aldrich (Shanghai) Trading Co., Ltd.). Malt extract, yeast extract, and peptone were purchased from AOBOX (Beijing Aoboxing Bio-Tech Co., Ltd.), and agar was purchased from Beijing BioDee Bio. Tech. Co., Ltd. (Beijing, China). Other chemicals used in present work were purchased from Tianjin Zhiyuan Chemical Reagent Co., Ltd. (Tianjin, China).

Lignocellulosic biomass

Populus beijingensis, obtained from Langfang city (Hebei province, China), was chopped into small pieces, air-dried, and ground with the micro-grinding machine FZ-102 into the particle size between 20- and 60-mesh.

Methods

Microbial culture

Flammulina velutipes CCMSSC 05331, *F. velutipes* FL 19, and *F. velutipes* CCMSSC 00103 were transferred to culture plate containing complete yeast agar medium (CYM, composed of glucose 20 g/L, peptone 2 g/L, yeast extract 2 g/L, $MgSO_4 \cdot 7H_2O$ 0.5 g/L, $K_2HPO_4 \cdot 3H_2O$ 1 g/L, KH_2PO_4 0.46 g/L, agar 15 g/L, and deionized water 1 L) and cultured at 26 °C for 7 days to activate the fungal strains.

Inoculum preparation

Five pieces with the diameter of 1.0 cm were punched by a round tool, then transferred into the 250-mL triangular flask containing 100 mL complete yeast medium (CYM without agar) and cultured at 26 ℃ with the rotation speed of 150 rpm to perform the seed liquid preparation process. Seven days later, the homogenization process of mycelium pellets in the triangular flask was completed using a hand-held homogenizer (Tianjin Hengao Technology Development Co., Ltd., Tianjin, China) for 2 min with the speed of 5000 rpm. The suspension was used in a subsequent process as an inoculum.

Process of submerged fermentation

The pH of fermentation medium used for laccase production of different *Flammulina velutipes* strains remained natural, and the formula was shown in Table 1. 100 mL of corresponding fermentation medium were added into 250-mL triangular flasks. Next, they were autoclaved at 121 ℃ for 30 min, and then 3 mL inoculum as mentioned above were added into every triangular flask. Next, all flasks were divided into two groups and cultured in a rotary shaker at 150 rpm with the temperature of 26 or 33 ℃, respectively.

Table 1. Details of Each Component of the Medium

Fermentation Medium	Alkali Lignin (g/L)	KH_2PO_4 (g/L)	Vitamin B1 (g/L)	Glucose (g/L)	Peptone (g/L)	*Populus beijingensis* (g/flask)
M1	3	3	0.03	-	-	-
M2	3	3	0.03	10	-	-
M3	3	3	0.03	-	2	-
M4	3	3	-	-	2	-
M5	3	3	0.03	10	2	-
M6	-	3	0.03	10	2	-
M7	-	3	0.03	-	-	3

Preparation of crude enzyme solution and laccase activity assay

Crude enzyme solution was prepared by filtering the fermentation liquor with a filter paper, and the filter liquor was centrifuged at 4 °C for 20 min with the speed of 12,000 rpm. The supernatant obtained after centrifugation was the crude enzyme solution that was used for the subsequent determination of laccase activity.

The laccase activity was determined by the method of Bourbonnais and Paice (1990), and the specific operation process was as described by An *et al*. (2018). Laccase activity was determined by measuring the changes in the absorbance when ABTS were oxidized at 420 nm ($\mathcal{E}_{420} = 3.6 \times 10^4\ M^{-1}\ cm^{-1}$). One unit of laccase activity was defined as the amount of laccase required to oxidize 1.0 μmol of ABTS per minute.

Biomass dynamics

To determine the biomass dynamics, mycelium pellets in the fermentation liquor were filtered by a filter paper, and washed three times with the deionized water to remove the fermentation medium. The washed mycelium pellets were finally dried at 60 °C to a constant weight.

Statistical analysis

To analyze the effects of fermentation medium, temperature, and strains on laccase activity, three-way analysis of variance (ANOVA) using the Tukey *post hoc* test was performed with SPSS 22.0 (PROC GLM, Armonk, NY, USA). All colorful figures were generated by the Origin 2016 software (OriginLab Corporation, Northampton, MA, USA).

RESULTS AND DISCUSSION

Laccase Activity and Mycelium Biomass in Different Fermentation Medium

It was found that laccase activity could be improved by the metal ions, lignocellulosic wastes, fungal species, and other factors. *Flammulina velutipes*, as a model white-rot fungus, had been shown to secrete laccase in previous studies (An *et al*. 2015, 2016; Janusz *et al*. 2015; Xie *et al*. 2017; Cesur *et al*. 2022a, 2022b). There are many studies on the effect of lignocellulosic materials or metal ions on laccase activity of white-rot fungi, including the species from the genus of *Flammulina* (An *et al*. 2016; Cesur *et al*. 2022a,b; Hasan *et al*. 2023; Kasirajan and Kamaraj 2023). Of course, alkali lignin has also been shown the capacity in increasing the laccase activity of *Ganoderma lucidum* and *Pleurotus ostreatus* (Sitarz *et al*. 2013; An *et al*. 2018). Based on this, the effect of alkaline lignin on laccase activity and mycelium biomass of *Flammulina velutipes* strains was investigated in this study.

The effect of fermentation medium, temperature, and strains on laccase activity was significant during the whole fermentation stage ($P < 0.001$). Maximum laccase activity of *Flammulina velutipes* CCMSSC 05331, *F. velutipes* FL 19, and *F. velutipes* CCMSSC 00103 detected in M1 at 26 °C was 26.39 ± 0.95 U/L, 37.25 ± 2.52 U/L, and 55.06 ± 5.34 U/L, appearing on the 5th, 3rd, and 8th day of fermentation, respectively (Fig. 1a, 1b, 1c).

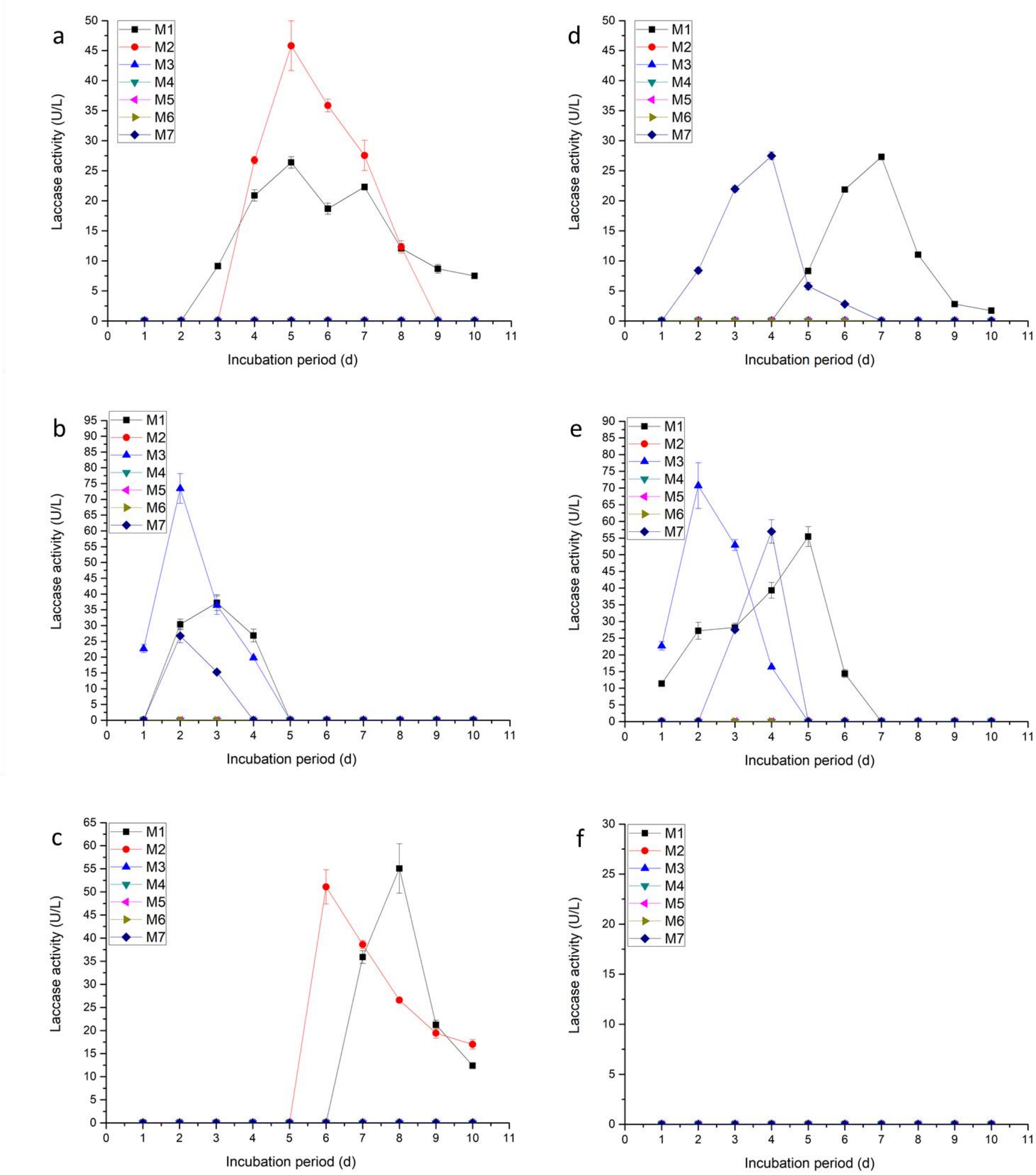

Fig. 1. Laccase activity of *Flammulina velutipes* strains fermented in different medium (a: *Flammulina velutipes* CCMSSC 05331 cultured at 26 °C; b: *F. velutipes* FL 19 cultured at 26 °C; c: *F. velutipes* CCMSSC 00103 cultured at 26 °C; d: *F. velutipes* CCMSSC 05331 cultured at 33 °C; e: *F. velutipes* FL 19 cultured at 33 °C; f: *F. velutipes* CCMSSC 00103 cultured at 33 °C). M1 = fermentation medium 1; M2 = fermentation medium 2; M3 = fermentation medium 3; M4 = fermentation medium 4; M5 = fermentation medium 5; M6 = fermentation medium 6; M7 = fermentation medium 7

Similarly, the maximum laccase activity of *Flammulina velutipes* CCMSSC 05331, *F. velutipes* FL 19, and *F. velutipes* CCMSSC 00103 detected in M2 at 26 °C was 45.81 ± 4.14 U/L, 73.42 ± 4.74 U/L, and 51.08 ± 3.70 U/L, appearing on the 5^{th}, 2^{nd}, and 6^{th} day of fermentation, respectively (Fig. 1a, 1b, 1c). Under the fermentation condition of M1 and M2, the mycelial biomass of *Flammulina velutipes* CCMSSC 05331, *F. velutipes* FL 19, and *F. velutipes* CCMSSC 00103 was 0.093 ± 0 g and 0.211 ± 0.009 g, 0.173 ± 0.002 g and 0.092 ± 0 g, and 0.026 ± 0 g and 0.060 ± 0.005 g (Fig. 2a, 2b, 2c). It is easy to find that the laccase activity value secreted by fungi had no direct linear relationship with its own mycelial biomass. In the whole, the maximum laccase value of *F. velutipes* strains in M 2 medium appeared earlier than that in M1 medium. It is worth mentioning that the *F. velutipes* strains reached the maximum mycelial biomass earlier with M2 than M1. However, the composition difference between M2 and M1 was that M2 contains glucose, a simple carbon source, which seemed to indicate that the presence of glucose helped *F. velutipes* strains to indirectly accelerate laccase to achieve maximum laccase activity by accelerating biomass accumulation. Of course, that does not mean that the presence of glucose leads to an increase in the total mycelial biomass. Kanwal and Reddy (2011) evaluated the effect of different carbon sources on ligninolytic activity secreted by *Morchella crassipes* and concluded that laccase activity variation depended on the carbon source used. Li *et al*. (2011) studied the mechanism of excess production of laccase under microbial interaction through the co-culture process of *Ganoderma lucidum* and *Candida* sp. HSD07A, and the results showed that nitrogen source, sulfur source, hydrolase, and inducers had no significant influence on laccase activity. In addition, it was apparent that glucose deprivation in the medium was also not the crucial reason why *G. lucidum* overproduces laccase, although it was able to improve laccase activity to a certain extent. Nutrient media optimization for simultaneous enhancement of the laccase and peroxidases production by coculture of *Dichomitus squalens* and *Ceriporiopsis subvermispora* was investigated by Kannaiyan *et al*. (2015), and the optimum nutrient levels were 1% glucose, 0.1% arabinose, 20 mM sodium nitrate, 0.27% casein, 0.31 mM $CuSO_4$, and 0.07 mM $MnSO_4$. An *et al*. (2018) found that the combination of alkaline lignin and glucose as an additional carbon source was conducive to the enhancement of laccase activity in *Pleurotus ostreatus*. Similarly, this study also found that glucose was beneficial to the improvement of laccase activity to some extent. Ottoni *et al*. (2016) investigated the effect of different carbon sources on laccase activity of *Trametes versicolor* and concluded that glycerol could be described as a good inducer to produce laccase. Thus, this also fully reflects the diversity of fungi using carbon nutrition.

Laccase activity of *F. velutipes* CCMSSC 05331 and *F. velutipes* CCMSSC 00103 fermented in M3, M4, M5, M6, and M7 at 26 °C could not be detected during the whole stage of fermentation, while the maximum laccase activity of *F. velutipes* FL 19 in M7 containing *Populus beijingensis* was 26.76 ± 2.21 U/L (Fig. 1a, 1b, 1c). Further, mycelial biomass of tested *F. velutipes* strains in M3, M4, M5, M6, and M7 could be measured (Fig. 2a, 2b, 2c). It was clear that although no laccase activity was detected in these *F. velutipes* strains under M5 medium, the mycelial biomass accumulated by these strains was very high, and respectively reached 0.400 ± 0.028 g (*F. velutipes* CCMSSC 05331), 0.375 ± 0.032 g (*F. velutipes* FL 19), and 0.210 ± 0.013 g (*F. velutipes* CCMSSC 00103). Compared with M2, M5 contained a simple nitrogen source of peptone. Thus, the presence of peptone increased the mycelial biomass of three tested strains. In addition, the ratio of carbon to nitrogen also affected the growth of mycelia.

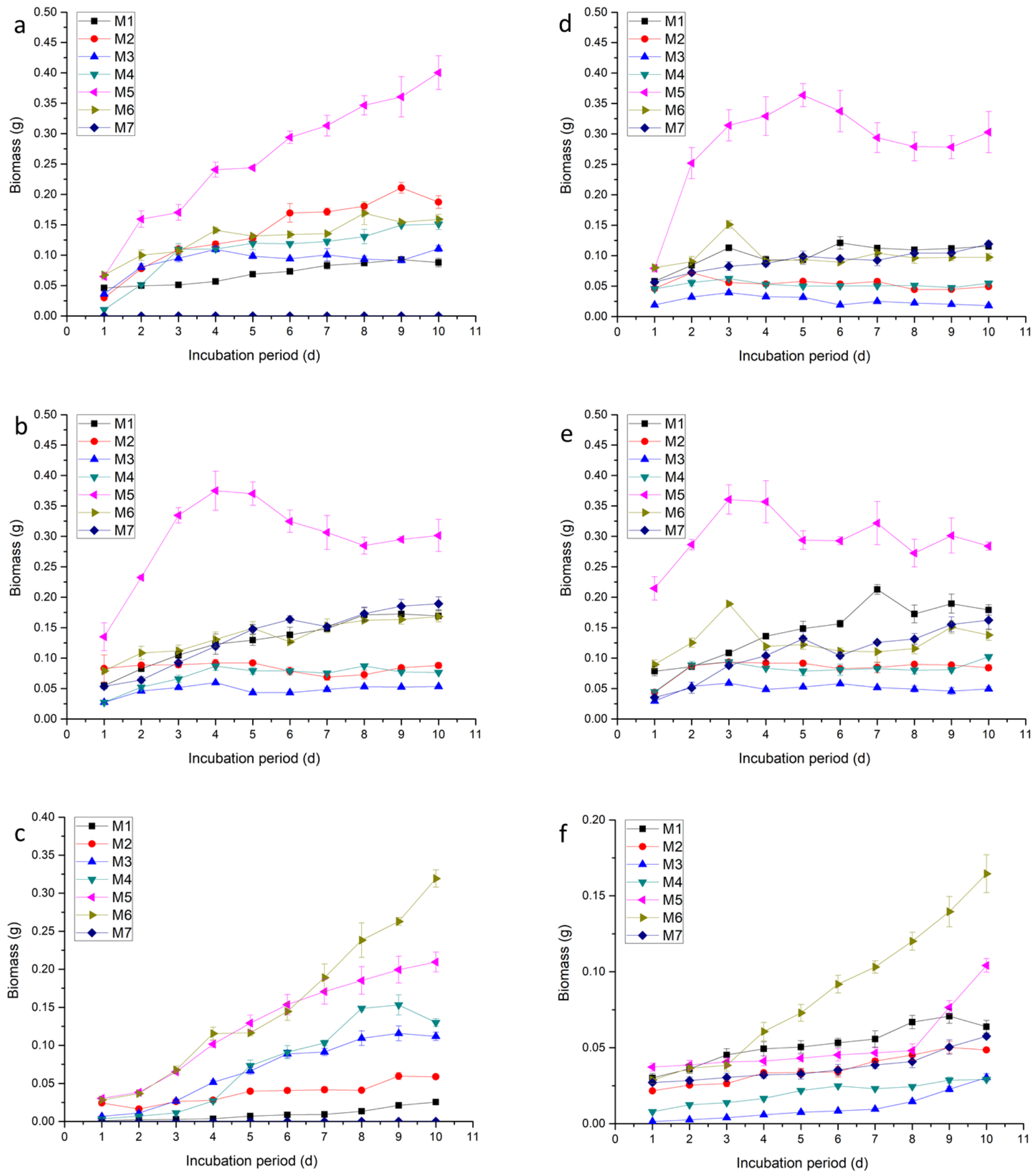

Fig. 2. Mycelium biomass of *Flammulina velutipes* strains fermented in different medium (a: *Flammulina velutipes* CCMSSC 05331 cultured at 26 °C; b: *F. velutipes* FL 19 cultured at 26 °C; c: *F. velutipes* CCMSSC 00103 cultured at 26 °C; d: *F. velutipes* CCMSSC 05331 cultured at 33 °C; e: *F. velutipes* FL 19 cultured at 33 °C; f: *F. velutipes* CCMSSC 00103 cultured at 33 °C) . M1 = fermentation medium 1; M2 = fermentation medium 2; M3 = fermentation medium 3; M4 = fermentation medium 4; M5 = fermentation medium 5; M6 = fermentation medium 6; M7 = fermentation medium 7

Colla *et al*. (2023) aimed to evaluate the carbon-to-nitrogen (C/N) ratios and nitrogen contents in sugarcane bagasse and soybean meal substrate formulations on mycelial growth, laccase activity, and mushroom production of *Lentinus crinitus* in solid-state cultivation, and found that mycelial growth, mushroom yield, biological efficiency, and biological efficiency-to-cultivation time ratio of *L. crinitus* were higher with the substrate of 1.2% nitrogen (C/N ratio of 36.6). Another aspect that cannot be ignored to account for the difference between M5 and M6 was that M5 contained alkaline lignin. Meanwhile, the mycelial biomass of *F. velutipes* strains was the highest in M5, indicating that alkaline lignin was helpful for accumulation of mycelial biomass. *F. velutipes* CCMSSC 05331 and *F. velutipes* CCMSSC 00103 did not grow in M7 at 26 °C, which was composed of *Populus beijingensis*, KH_2PO_4, and vitamin B1. It can be seen that complex lignocellulosic materials with lignin content were not conducive to the growth of mycelia. When fermented at 33 °C, laccase activity of *Flammulina velutipes* CCMSSC 05331 could be measured in M1 and M7, and laccase activity of *F. velutipes* FL 19 could be measured in M1, M3, and M7, while no laccase activity of *F. velutipes* CCMSSC 00103 was detected in all fermentation medium (Fig. 1d, 1e, 1f). In addition, the mycelial biomass of *F. velutipes* CCMSSC 05331 and *F. velutipes* FL 19 was the highest in M5 at 33 °C, while maximum mycelial biomass of *F. velutipes* CCMSSC 00103 was obtained from M6 (Fig. 2d, 2e, 2f).

Xu *et al*. (2020) found that the laccase production of *Trametes versicolor* obtained from the optimizing culture medium with tea residues was 25.7 U/g dry substrate, resulting in a 4.0-fold increase compared with the laccase production from unoptimized culture medium. Gou *et al*. (2022) found that corn stover could replace glucose as a carbon source to promote laccase production. Of course, other lignocellulosic materials, such as cassava waste, corn straw, wheat straw, straw, and even urban food waste, have also been reported to affect the secretion of laccase by fungi (Wang *et al*. 2015; Unuofin *et al*. 2019; Kumar *et al*. 2022). All the above studies have shown that the presence of lignocellulosic materials can promote fungal laccase activity, but it is based on the accumulation of mycelial biomass.

Laccase Activity and Mycelium Biomass of Different *Flammulina velutipes* Strains

There has been continuous development of new laccase-producing strains and evaluation of their laccase-producing capacity. Therefore, a large number of white-rot fungi have been shown to produce laccase (Gonzalez-Gonzalez *et al*. 2023).

Maximum laccase activity of *Flammulina velutipes* CCMSSC 05331, *F. velutipes* FL 19, and *F. velutipes* CCMSSC 00103 was 45.81 ± 4.14 U/L fermented in M2 at 26 ℃, 73.42 ± 4.74 U/L fermented in M 2 at 26 ℃, and 55.06 ± 5.34 U/L fermented in M1 at 26 ℃ (Fig. 1). Similarly, maximum laccase activity of *F. velutipes* FL 19 at 33 ℃ was nearly 2.59-fold larger than that from *F. velutipes* CCMSSC 05331, and laccase activity of *F. velutipes* CCMSSC 00103 was not measured in all test fermentation media during the whole fermentation stage. Thus, the laccase secreting ability of *F. velutipes* FL 19 was higher than that of *F. velutipes* CCMSSC 05331 and *F. velutipes* CCMSSC 00103. Of course, the media in which these strains were able to produce the enzyme were also inconsistent.

Janusz *et al*. (2015) investigated the laccase production and metabolic diversity among 12 *Flammulina velutipes* strains and showed different laccase secretion ability of twelve strains. An *et al*. (2016) analyzed the laccase activity of 13 strains belonging to the genus of *Flammulina* fermented in different culture media and found that the presence of simple carbon and nitrogen sources increased the maximum laccase enzyme activity. Meanwhile, there were obvious differences in laccase secretion ability among different *Flammulina* strains, which was in agreement with the present study. Similarly, different strains of other fungi, such as *Pleurotus* and *Trametes*, have the same characteristics (Mallak *et al*. 2021; Melanouri *et al*. 2022).

In addition to the differences in laccase production capacity, there were also differences in mycelial biomass accumulation among these *Flammulina velutipes* strains (Fig. 2). *F. velutipes* CCMSSC 05331 and *F. velutipes* CCMSSC 00103 can grow in M1, M2, M3, M4, M5, and M6, at 26 °C and M1, M2, M3, M4, M5, M6, and M7 at 33 °C (Fig. 2). However, the mycelial biomass of *F. velutipes* FL 19 could be detected in all tested fermentation medium at 26 and 33 °C (Fig. 2). Based on this, the adaptability of *F. velutipes* CCMSSC 05331 and *F. velutipes* CCMSSC 00103 to the medium was similar. However, the maximum value of mycelial biomass of *F. velutipes* CCMSSC 05331 fermented at 26 and 33 °C was higher than the maximum mycelial biomass of *F. velutipes* CCMSSC 00103. Moreover, it is not difficult to find that *F. velutipes* CCMSSC 05331 had the largest mycelial growth at 26 and 33 °C when compared with the other two strains, which also reflects the differences in nutrient utilization and metabolism among different strains of the same species.

Laccase Activity and Mycelium Biomass with Different Temperature

Maximum laccase activity of *Flammulina velutipes* CCMSSC 05331, *F. velutipes* FL 19, and *F. velutipes* CCMSSC 00103 at 26 °C was higher than that measured at 33 °C (Fig. 1). Thus, *F. velutipes* strains were more suitable for secreting laccase at 26 °C. However, the mycelia growth of three *F. velutipes* strains did not show the consistency of laccase at different temperatures, and the mycelia biomass varied under different medium conditions. Nevertheless, the maximum mycelium biomass for each *F. velutipes* strain was still present at a culture temperature of 26 °C, but not in the same medium as the maximum laccase activity. Yang *et al*. (2020) discovered a thermo-active laccase isoenzyme from *Trametes trogii*, and the optimal temperature after purification was found to be 60 °C. Sun *et al*. (2021) investigated the extracellular laccase of the litter decomposing fungus *Gymnopus luxurians* and demonstrated that an optimum temperature range of *G. luxurians* was 55 to 65 °C. This seems to indicate that the optimum temperature of the purified laccase is not consistent with that of the crude laccase protein.

CONCLUSIONS

1. Among the three strains of *Flammulina velutipes*, the laccase secretion capacity of *F. velutipes* FL 19 was clearly better than that of *F. velutipes* CCMSSC 05331 and *F. velutipes* CCMSSC 00103.
2. The presence of alkali lignin was useful for improving laccase activity secreted by *Flammulina velutipes*. The effect of alkali lignin on improving laccase activity even exceeded that of *Populus beijingensis*.
3. There was no linear relationship between the content of mycelium biomass and laccase activity of *Flammulina velutipes*.
4. *F. velutipes* strains were more suitable for secreting laccase at 26 °C due to the maximum laccase activity measured at 26 °C was higher than that measured at 33 °C.

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