Enhanced Laccase Activity of White Rot Fungi Induced by Different Metal Ions under Submerged Fermentation

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Submerged fermentation with single or mixed metal ions as inducers was used for laccase production from white rot fungi. Mixed metal ions were used for the first time as inducers for *Pleurotus ostreatus* and *Flammulina velutipes* to enhance laccase activity. The maximum laccase activity from *P. ostreatus* in basal media, metal ion media 1 containing copper ion, metal ion media 2 containing manganese ion, metal ion media 3 containing manganese and copper ions, metal ion media 4 containing ferrous ion, metal ion media 5 containing manganese and ferrous ions, metal ion media 6 containing ferrous and copper ions, and metal ion media 7 containing manganese, copper and ferrous ions were, respectively, approximately 21.5-fold, 4.7-fold, 14.9-fold, 16.9-fold, 4.0-fold, 11.0-fold, 12.7-fold, and 24.8-fold higher than that from *F. velutipes*. The combination of copper and manganese ions as inducers was superior to that of a single copper ion or manganese ion. The maximum laccase activity of *P. ostreatus* rose in media containing manganese and copper ions. The single copper ion as the inducer for enhancing laccase activity was more suitable for *F. velutipes*. These findings are helpful in selecting the appropriate single metal ion or mixed metal ions to enhance laccase activity.

Keywords: White rot fungi; Laccase activity; Metal ions; Submerged fermentation

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INTRODUCTION

Laccase (EC 1.10.3.2, *p*-diphenol: dioxygen oxidoreductase) belongs to a group of polyphenol oxidases containing copper atoms in a catalytic center (Baldrian 2006). Laccase has the ability to oxidize phenols and aromatic amines and reduce molecular oxygen to water. Laccase was first described by Yoshida (1883), and it has been detected in plants, fungi, bacteria, and insects (Mayer and Staples 2002). Many fungi in Basidiomycota and Ascomycota have been shown to secrete laccase (An *et al.* 2019). Among them, laccases are typical of wood rot basidiomycetes and a related group of litter-decomposing fungi that can degrade lignin (An *et al.* 2018, 2019). Almost all species of white rot fungi have the ability to secrete laccase (Singh Arora and Kumar Sharma 2010; Wang *et al.* 2019), and laccase production is higher in white rot fungi (Basidiomycetes) as compared with bacteria (Wang *et al.* 2019). The genus *Pleurotus* (Fr.) P. Kumm. and genus *Flammulina* P. Karst., both belonging to Basidiomycete, are typical and important groups of white rot fungi and laccase producers (Park *et al.* 2014; Janusz *et al.* 2015; An *et al.* 2018).

However, there are relatively few studies concerning the activity of laccase secreted by the genus *Flammulina*. Because of their high catalytic efficiency, laccases are used for technical applications in various aspects of the industrial and biotechnological fields, such as for the decolorization of synthetic dyes, improving fiber properties, energy exploitation, detoxification of environmental pollutants, pulp bleaching in the paper industry, bio-synthesis, bio-detection, conversion of aromatic compounds, and wastewater treatment (Kudanga and Le Roes-Hill 2014; Mogharabi and Faramarzi 2014; Bilal *et al.* 2019; Deska and Kończak 2019). In addition, it is also used in fast-moving consumer goods (FMCG) and cosmetics; in the beverage and food industry for the removal of phenolic compounds in juice and wine, and to increase the strength of gluten structures in dough or baked products; in pharmaceutical industries; in nanobiotechnology; and for other miscellaneous applications (Fernández-Fernández *et al.* 2013; Upadhyay *et al.* 2016; Ba and Kumar 2017; Bertrand *et al.* 2017; Singh and Arya 2019; Unuofin *et al.* 2019; Zerva *et al.* 2019).

Given these diverse applications, laccases have been intensively studied since the 19th century. However, the application of laccase in biotechnological processes has been limited because of high production costs resulting from low enzyme activity and low yield. Therefore, increasing research attention has been paid to effective laccase production strategies associated with increased activity and reduced costs (Cardona *et al.* 2010; An *et al.* 2018). The important starting point of these efforts examines novel productive strains for large-scale laccase production with minimum energy consumption (Cardona *et al.* 2010; Agrawal *et al.* 2018).

The production of extracellular laccases from white rot fungi depends on species, strain, and the kinds of carbon and/or nitrogen sources, as well as their concentration and the ratio, metals, and fermentation methods (Boran and Yesilada 2011; An *et al.* 2018; Filipe *et al.* 2019; Akpinar and Urek 2020; Han *et al.* 2020b; Rajavat *et al.* 2020). The main fermentation methods usually used are solid-state fermentation (SSF), submerged fermentation (SmF), and sequential solid-state and submerged cultivation fermentation (Téllez-Téllez *et al.* 2008; An *et al.* 2016b).

Among them, SSF and SmF are most commonly used. Solid-state fermentation is the direct interaction between fungi and culture substrates under the condition of low water content. Submerged fermentation promotes the growth of fungi in a liquid culture with a simple carbon and nitrogen source or a complex lignocellulosic biomass as the substrate. The oscillating culture during SmF can also enhance the supply of oxygen (Ikubar *et al.* 2018; Han *et al.* 2020b). In addition, different species or strains in the same species of fungi have different abilities for laccase secretion (Elisashvili *et al.* 2008; Janusz *et al.* 2015; An *et al.* 2016a; Han *et al.* 2020a). To some extent, this difference also reflects the importance of developing new productive strains. Thus, it is also natural to select a suitable culture medium for these new productive strains.

The effects of carbon and/or nitrogen sources and the concentration ratio to laccase production secreted by white rot fungi have also been investigated. Because lignocellulosic biomass is a renewable and complex material, it has become a popular research object for lignocellulolytic enzyme production in the fungi cultivation process (An *et al.* 2016b; Han *et al.* 2017, 2018; Filipe *et al.* 2019; Huang *et al.* 2019; Leite *et al.* 2019; Akpinar and Urek 2020; Rajavat *et al.* 2020). However, the regulation of laccase expression by metals has only recently been reported. Previous studies have shown that copper is one of the well-known inducers of laccase activity among various metals (Cd^{2+}, Ag^{+}, Hg^{2+}, Mn^{2+}, and Cu^{2+}) (Palmieri *et al.* 2000; Baldrian and Gabriel 2002; Soden and Dobson 2003; Liu *et al.* 2009; Mäkela *et al.* 2013; An *et al.* 2016a). Baldrian and Gabriel (2002) reported that heavy

metals are an important group of enzyme activity inducers and the extra addition of Cd^{2+} and Cu^{2+} as inducers can increase the activity of laccase obtained from *P. ostreatus* grown in a nitrogen-limited medium. An *et al.* (2016a) reported that generally the presence of copper could increase laccase activity obtained from strains belonging to *Flammulina*. Zhou *et al.* (2017) reported that high concentrations of the Mn^{2+} ion exhibited weak inhibition of laccase and Fe^{2+} ion showed strong inhibition of laccase activity. Zhuo *et al.* (2017) reported that Fe^{2+} ion from 1 to 2 mM stimulated the production of extracellular laccase from *P. ostreatus* HAUCC 162. However, the existing studies on the effect of metal ions on fungal laccase secretion mostly focus on the single ion induction. Few studies have taken into consideration the induction of mixed metal ions.

The present study mainly discussed the effects of copper, ferrous, and manganese ions on the laccase secretion of white rot fungi *Pleurotus* sp. and *F. velutipes* in submerged fermentation. The mixing of these metal ions to induce tested fungi secretion of laccase was also investigated. The results aim to provide the basis for the further purification of laccase, the selection of appropriate single metal ion or mixed metal ions to enhance laccase activity, and the optimization of integrated industrial laccase production.

EXPERIMENTAL

Materials

The two strains of white rot fungi, from the Institute of Microbiology, Beijing Forestry University (Beijing, China), used in this study were *Pleurotus* sp. CCEF 89 and *Flammulina velutipes* CCMSSC 00114. All fungi were maintained on complete yeast medium (CYM) agar (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, and agar 20 g/L) at 4 °C. All microorganisms were transferred to new CYM agar plates and incubated at 26 °C for 7 days.

Methods

Inoculum preparation

Inoculates of *Pleurotus* sp. CCEF 89 and *F. velutipes* CCMSSC 00114 with a diameter of 5 mm were prepared using a perforator and transferred to 250-mL Erlenmeyer flasks with 100 mL CYM liquid medium for culture. The whole process was completed in a rotary shaker at 26 ℃ with a speed of 150 rpm. After 7 days, mycelial pellets were harvested and homogenized with a laboratory blender (Tianjin hengao Technology Development Co., Ltd, Tianjin, China) for 2 min at 5000 rpm. The resulting suspension was used as an inoculum.

Submerged fermentation with different metal ion mediums

Submerged fermentation (SmF) was completed in 250-mL Erlenmeyer flasks filled with 100 mL of liquid media. The basal medium, named BM, was prepared using the following chemicals: 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, and 0.46 g/L KH_2PO_4. Seven different metal ion media were used in the experiments, named MM 1, MM 2, MM 3, MM 4, MM 5, MM 6, and MM 7. The MM1 medium was composed of BM media and copper ions with a final ion concentration of 2 mM. The MM2 medium was composed of BM media and manganese ions with a final ion concentration of 2 mM. The MM3 medium was composed of BM

medium, copper, and manganese ions with each final ion concentration of 2 mM. The MM4 medium was composed of BM media and ferrous ions with a final ion concentration of 2 mM. The MM5 medium was composed of BM media, ferrous and manganese ions with each final ion concentration of 2 mM. The MM6 medium was composed of BM medium, ferrous and copper ions with each final ion concentration of 2 mM. The MM7 media was composed of BM medium, ferrous, manganese, and copper ions with each final ion concentration of 2 mM. Then 3 mL of inoculum was added into every 250-mL Erlenmeyer flasks filled with 100 mL of different liquid media. All flasks were incubated at 26 °C.

To determine the laccase activity dynamics under different metal ion medium conditions in SmF, *Pleurotus* sp. cultures and *F. velutipes* cultures from the first day to the twentieth day were measured.

Assay for laccase activity

Culture liquids were obtained by filtering the cultures through Whatman No. 1 filter paper. The filtrates were then centrifuged (4 ℃, 12000 rpm, 15 min), and the supernatants were used for measurement of enzyme activity.

Laccase (EC 1.10.3.2) activity was determined by the changes in the absorbance at 420 nm related to the rate of oxidation of 1 mM 2,2'-azinobis-[3-ethyltiazoline-6-sulfonate] (ABTS) in 50 mM of sodium acetate buffer (pH 4.2). The assay mixture was measured in a 3-mL cuvette with 50 μL of adequately diluted culture liquid (Wolfenden and Wilson 1982). One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 μmol of ABTS per minute ($\varepsilon_{420} = 3.6 \times 10^4$ M^{-1} cm^{-1}).

All measurements were derived from three independent experiments and the mean values were taken. The standard deviations for the experiments were less than ± 10%.

Data analysis

To examine the effects of fungal species and metal ions on laccase activities, two-way analysis of variance followed by the Tukey *post hoc* test was applied to these variables, with fungal species and metal ions being fixed factors (PROC GLM, IBM SPSS software, version 22.0, Armonk, NY, USA). All statistical figures were generated using the program Origin Pro 2016 (OriginLab Corporation, Northampton, MA, USA).

Genomic DNA extraction, polymerase chain reaction, and sequencing

Mycelia for DNA extraction were grown on CYM agar medium for 7 days, and samples were obtained from the surfaces of CYM agar medium by scraping. The total genomic DNA of the mycelia was extracted by a cetyl trimethylammonium bromide rapid plant genome extraction kit (Aidlab Biotechnologies Co., Ltd., Beijing, China) according to manufacturer's instructions with some modifications (An *et al.* 2016a; Han *et al.* 2016). The ITS regions were amplified with primer pairs ITS5 and ITS4 (Han *et al.* 2016). The polymerase chain reaction (PCR) cycling schedule for ITS included an initial denaturation at 95 °C for 3 min, followed by 35 cycles at 94 °C for 40 s, 54 ℃ for 45 s, 72 °C for 1 min, and a final extension at 72 °C for 10 min (Han *et al.* 2016). The PCR products were purified and sequenced at Beijing Genomics Institute (Beijing, China), with the same primers. The newly generated sequence was deposited at GenBank.

RESULTS AND DISCUSSION

Molecular Biological Results

The test strain of *Pleurotus* sp. CCEF 89 was identified by molecular biology as *P. ostreatus* and GenBank no. MT490883. Another test strain of *F. velutipes* CCMSSC 00114 had been accurately identified with ITS sequence by An *et al.* (2016a).

Effect of Metal Ion on Laccase Production

Laccase is known as an inducible enzyme and necessary for degrading lignin by many white rot fungi such as *Pleurotus ostreatus*, *Trametes hirsute*, *T. versicolor*, *Ceriporiopsis subvermispora*, *Ganoderma lucidum*, and *Cerrena unicolor* (De Souza-Cruz *et al.* 2004; Elisashvili and Kachlishvili 2009; Kachlishvili *et al.* 2014; Singh *et al.* 2019; Han *et al.* 2020a; Huang *et al.* 2020).

Table 1. Effects of Species, Media, and Species × Media Interactions on the Laccase Activities of *P. ostreatus* and *F. velutipes* (Two-Way ANOVA)

Incubation Period (d)	Species	Media	Species × Media
1	4502.959***	359.301***	359.301***
2	4046.308***	385.165***	225.868***
3	2484.533***	505.936***	425.336***
4	7570.275***	1563.487***	1273.240***
5	21415.928***	5226.109***	5081.993***
6	1770.441***	640.617***	737.742***
7	1292.678***	548.015***	814.190***
8	2929.213***	809.553***	744.297***
9	3332.540***	1632.499***	1038.962***
10	1247.443***	534.436***	310.005***
11	2947.534***	622.487***	397.342***
12	2511.831***	916.882***	788.131***
13	3030.618***	1150.942***	1102.790***
14	5078.174***	2094.865***	2003.303***
15	1276.414***	449.144***	449.502***
16	6633.866***	1572.499***	1569.769***
17	3321.341***	662.222***	656.386***
18	4198.908***	978.865***	976.100***
19	1221.098***	292.986***	292.989***
20	787.029***	267.989***	267.996***
*Note: df = 1, 7, 7; *P < 0.05, **P < 0.01, ***P < 0.001			

Most studies have focused on lignocellulosic materials' effects on fungal lignocellulolytic enzyme production because lignocellulosic biomass is one of the most renewable and abundant eco-friendly resource on earth (Han *et al.* 2020a). However, metal ions are also an important factor in inducing the secretion of fungal laccase (Galhaup *et al.* 2002; Janusz *et al.* 2015; An *et al.* 2016a). In addition, previous studies mainly focused on the effect of a single metal on laccase activity from white rot fungi. The examination of the effect of mixed metal ions on laccase activity from white rot fungi is rare. In the present study, single metal ions and mixed metal ions were used as the inducer to study the effect

of metal ions on laccase production for tested white rot fungi under submerged fermentation. As shown in Table 1, the effect of metal ions on the laccase activities of *P. ostreatus* CCEF 89 and *F. velutipes* CCMSSC 00114 were highly significant throughout the testing process ($P < 0.001$).

Clearly, the trend of laccase activity from *P. ostreatus* CCEF 89 with BM media cultivated was on the rise for the first 5 days, and then it went down (Fig. 1). The laccase activity for *P. ostreatus* CCEF 89 ranged from 27.22 ± 0.48 U/L to 1220.74 ± 62.51 U/L, and the maximum laccase activity occurred on the fifth day (Table 2). In addition, *P. ostreatus* CCEF 89 was at a low level of laccase activity for most of the fermentation period. Using MM 1 medium, which contained copper ions as enzyme-producing medium, *P. ostreatus* CCEF 89 secreted the higher activity in the middle and late stage of fermentation (Fig. 1). The laccase activity ranged from 24.48 ± 2.49 U/L to 11661.57 ± 659.85 U/L, and the maximum laccase activity occurred on the sixteenth day (Table 2). Furthermore, the maximum laccase activity from *P. ostreatus* CCEF 89 in MM 1 medium was nearly 9.6-fold higher than that in BM medium. The results were similar to previous studies and seemed to directly indicate the good ability of copper ions to induce the secretion of laccase in *P. ostreatus* (Baldrian and Gabriel 2002; Liu *et al.* 2009; Mäkela *et al.* 2013; An *et al.* 2016a). Using MM 2 medium, which contained manganese ions as enzyme-producing medium, the laccase activity ranged from 48.06 ± 3.54 U/L to 1796.94 ± 51.03 U/L (Fig. 1). The maximum laccase activity appeared on the eighth day (Table 2) and was nearly 1.5-fold higher than that in BM medium. That increase suggested that manganese ions contributed to the laccase secretion of *P. ostreatus*, but the difference was large compared with the effect of copper ions. The maximum laccase activity in MM 1 medium was 6.5-fold higher than in MM 2 medium (Table 2). Moreover, manganese ions promoted laccase secretion of *P. ostreatus* in present study, which was also different from a previous study (Zhou *et al.* 2017). Using MM 3 medium, which contained manganese and copper ions as enzyme-producing media, the production of laccase exceeded 6700 U/L after 10 days of the fermentation process (Fig. 1). Although there was a small peak at approximately 1628.61 ± 89.97 U/L on the seventh day, the laccase activity was relatively lower than the maximum value (21966.67 ± 2194.50 U/L) that occurred on the twentieth day. In this condition, maximum laccase activity was nearly 18.0-fold, 1.9-fold, and 12.2-fold than that observed in BM medium, MM 1 medium, and MM 2 medium, respectively (Table 2). In addition, the mixed induction effect of the manganese ion and the copper ion was clearly greater than that of copper ion or manganese ion alone. Additionally, it was relatively higher than previous studies on the laccase activity from *P. ostreatus* (Baldrian and Gabriel 2002; Téllez-Téllez *et al.* 2008; Liu *et al.* 2009; Zhu *et al.* 2016). Using MM 4, which contained ferrous ions as enzyme-producing media, the level of laccase activity during whole fermentation process was low (Fig. 1). The laccase activity ranged from 33.33 ± 2.92 U/L to 317.59 ± 16.15 U/L (Fig. 1). The maximum laccase activity appeared on the fifth day (Table 2) and was approximately 3.8-fold and 69.2-fold lower than that observed, respectively, in BM medium and MM 3 medium. Previous studies indicated that ferric ion was disadvantaged of laccase secretion from *P. ostreatus* (Liu *et al.* 2009). Results of the present study clearly indicated that the ferrous ions with the concentration of 2 mM were not conducive to the secretion of laccase for *P. ostreatus*.

Using MM 5 medium, which contained manganese and ferrous ions as enzyme-producing media, the level of laccase activity during whole fermentation process was similarly low compared with that in MM 4 medium (Fig. 1). The maximum laccase activity in MM 5 medium was 618.89 ± 14.77 U/L on the eleventh day (Table 2) and approximately

2.0-fold and 35.5-fold lower than that observed, respectively, in BM medium and MM 3 medium, but the maximum laccase activity was approximately 1.9-fold higher than that in MM 4 medium. This result again indicated that the manganese ion contributed to laccase activity, in spite of some studies that had shown that the manganese ion was detrimental to laccase secretion (Liu *et al*. 2009). Different species or strains of the same species had different adaptability to metal ions (Scheel *et al*. 2000; An *et al*. 2016a; Zhuo *et al*. 2017).

Using MM 6 medium, which contained ferrous and copper ions as enzyme-producing media, the trend of laccase activity demonstrated notable changes (Fig. 1). The laccase activity ranged from 18.02 ± 1.20 U/L to 4223.61 ± 355.37 U/L (Fig. 1). The maximum laccase activity appeared at the 20th day (Table 2) and was approximately 3.5-fold and 13.3-fold higher than that observed in BM medium and MM 4 medium, respectively. However, the maximum laccase activity was approximately 2.8-fold lower than that in MM 1 medium. The promotion of copper ions and the inhibition of ferrous ions for laccase activity were also proven by the results.

Using MM 7 medium, which contained ferrous, manganese, and copper ions as enzyme-producing media, the laccase activity ranged from 75.78 ± 2.36 U/L to 14056.48 ± 783.41 U/L (Fig. 1). The maximum laccase activity appeared on the seventeenth day (Table 2) and was approximately 11.5-fold, 1.2-fold, 7.8-fold, and 44.3-fold higher than that observed in BM medium, MM 1 medium, MM 2 medium, and MM 4 medium, respectively. However, the maximum laccase activity was approximately 1.56-fold lower than that in MM 3 medium.

Table 2. Maximum Laccase Activities, Media, Species, and Occurrence Time

Maximum Laccase Activity (U/L)	Media	Species	Occurrence time (day)
1220.74 ± 62.51	BM	*P. ostreatus*	5
56.85 ± 0.89	BM	*F. velutipes*	7
11661.57 ± 659.85	MM 1	*P. ostreatus*	16
2483.06 ± 17.49	MM 1	*F. velutipes*	7
1796.94 ± 51.03	MM 2	*P. ostreatus*	8
120.94 ± 11.31	MM 2	*F. velutipes*	6
21966.67 ± 2194.50	MM 3	*P. ostreatus*	20
1300.93 ± 58.02	MM 3	*F. velutipes*	10
317.59 ± 16.15	MM 4	*P. ostreatus*	5
78.44 ± 1.92	MM 4	*F. velutipes*	8
618.89 ± 14.77	MM 5	*P. ostreatus*	11
56.39 ± 1.42	MM 5	*F. velutipes*	8
4223.61 ± 355.37	MM 6	*P. ostreatus*	20
331.39 ± 1.40	MM 6	*F. velutipes*	8
14056.48 ± 783.41	MM 7	*P. ostreatus*	17
567.36 ± 27.01	MM 7	*F. velutipes*	10
Data are presented as mean ± standard deviation for triplicates			

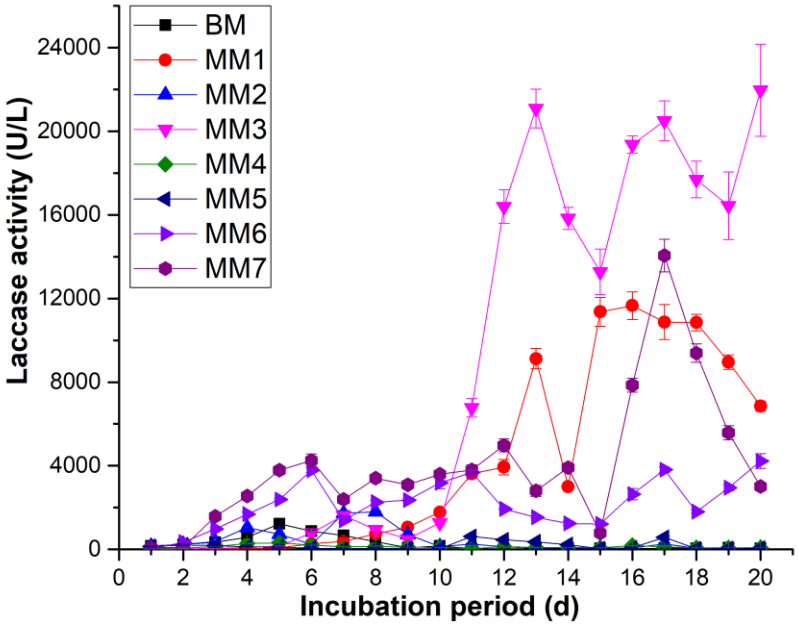

Fig. 1. The effect of different media on the laccase production by *Pleurotus ostreatus* CCEF 89; the average values were calculated from individual measurements for each of the three parallel cultures.

The laccase activity from *F. velutipes* CCMSSC 00114 in BM medium was low throughout the fermentation phase (Fig. 2). The maximum laccase activity was only 56.85 ± 0.89 U/L on the seventh day (Table 2). Using MM 1 medium, which contained copper ions as enzyme-producing medium, *F. velutipes* CCMSSC 00114 secreted the higher activity (2483.06 ± 17.49 U/L) on the seventh day (Fig. 1, Table 2) and the laccase activity was approximately 43.7-fold higher than that in BM medium. The result indicated that the presence of copper ions could promote the laccase activity of *F. velutipes* and was similar to the above-mentioned results of *P. ostreatus* CCEF 89. Meanwhile, previous study showed that laccase activity of *F. velutipes* CCMSSC 00114 from basal medium that contained cottonseed hull and copper ions was higher than that from Cu(–) medium that contained cottonseed hull (An *et al.* 2016a). Similarly, Janusz *et al.* (2015) reported that the final concentration of copper ions at 10 μM could promote laccase production in some strains of *F. velutipes*, but inhibited laccase production in other strains. These studies' results were similar to this study. Using MM 2 medium, which contained manganese ions as enzyme-producing media, the maximum laccase activity was 120.94 ± 11.31 U/L on the sixth day (Table 2) and approximately 2.1-fold higher than that in BM medium. However, the maximum laccase activity was approximately 20.5-fold lower than that in MM 1 media. The presence of manganese ions could promote the laccase activity of *F. velutipes*, while the induction effect of manganese ions was not as good as that of copper ions. Zhou *et al.* (2017) reported that high concentrations of the Mn^{2+} ion exhibited weak inhibition of laccase. But previous studies on the effect of manganese ions on ligninolytic enzymes mainly focused on their effects on manganese peroxidase or versatile peroxidase and relatively few on laccase (Cohen *et al.* 2002; Knop *et al.* 2014). Using MM 3 medium, which contained manganese and copper ions as enzyme-producing media, the maximum

laccase activity was 1300.93 ± 58.02 U/L on the tenth day (Table 2) and approximately 22.9-fold and 10.8-fold higher than that in BM medium and MM 2 medium. However, the maximum laccase activity was approximately 1.9-fold lower than that in MM 1 medium. Using MM 4 medium, which contained ferrous ions as enzyme-producing media, the maximum laccase activity was 78.44 ± 1.92 U/L (Fig. 2) on the eighth day and approximately 1.4-fold than that in BM medium. It was noteworthy that the presence of ferrous ions was advantageous to the laccase activity for *F. velutipes*, but the induction effect of ferrous ions was not as good as that of copper ions or manganese ions. That effect was also different from *P. ostreatus*. However, Zheng *et al.* (2017) indicated that the presence of ferrous ions inhibited the activity of purified laccase secreted by *Trametes orientalis*. The probable reason could be that the effect of ferrous ions on laccase secretion by different fungi was different. Using MM 5 medium, which contained manganese and ferrous ions as enzyme-producing media, the maximum laccase activity was 56.39 ± 1.42 U/L (Fig. 2) on the eighth day and similar to that in BM medium. Using MM 6 medium, which contained ferrous and copper ions as enzyme-producing media, the maximum laccase activity was 331.39 ± 1.40 U/L on the eighth day and approximately 5.8-fold higher than that in BM medium. Using MM 7 medium, which contained ferrous, manganese, and copper ions as enzyme-producing media, the maximum laccase activity was 567.36 ± 27.01 U/L on the tenth day and approximately 10.0-fold higher than that in BM medium.

Clearly, this was the first study on laccase activity with mixed metal ions as an inducer in white rot fungi and the results showed potential research value for further application in industry. Different white rot fungi will most likely choose a mixture of different metal ions according to their preferences. In this study, *P. ostreatus* CCEF 89 preferred a mixture of copper ions and manganese ions used as enzyme-producing media to single ion induction. However, *F. velutipes* CCMSSC 00114 was more suitable for the single copper ion as inducer. Meanwhile, a mixture of copper ions and manganese ions was also advantaged for *F. velutipes* CCMSSC 00114 secreting laccase.

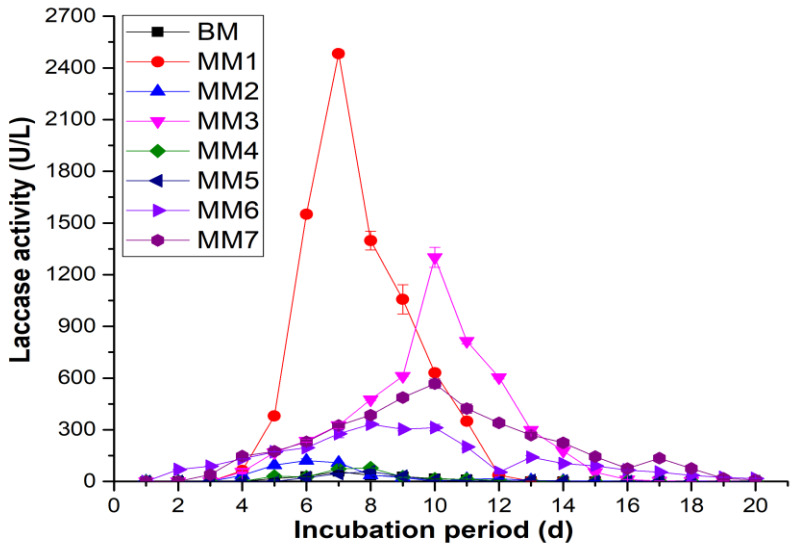

Fig. 2. The effect of different media on the laccase production by *Flammulina velutipes* CCMSSC 00114; the average values were calculated from individual measurements for each of the three parallel cultures.

Effect of species on Laccase Production

P. ostreatus CCEF 89 and the *F. velutipes* CCMSSC 00114 demonstrated notably different levels of laccase activity in these media (Fig. 1). The varying media also affected the difference in laccase secretion ability of different species (Elisashvili *et al.* 2008; An *et al.* 2016a, 2018). Overall, laccase activities of *P. ostreatus* were higher than those of *F. velutipes*. In terms of BM media, MM 1, MM 2, MM 3, MM 4, MM 5, MM 6, and MM 7, laccase activity from *P. ostreatus* CCEF 89 was approximately 21.5-fold, 4.7-fold, 14.9-fold, 16.9-fold, 4.0-fold, 11.0-fold, 12.7-fold, and 24.8-fold higher than that from *F. velutipes* CCMSSC 00114 (Table 2). Meanwhile, the time of maximum laccase activity from *F. velutipes* occurred earlier than that from *F. velutipes* under different fermentation media. Clearly, the induction of copper and manganese ions was conducive to laccase activity from *P. ostreatus*, but the induction of ferrous ion inhibited laccase activity. However, the induction of copper ions, ferrous ions, and manganese ions were all conducive to laccase activity from *F. velutipes*. Previous studies demonstrated that copper ions promoted the laccase activity of *F. velutipes*, while the role of ferrous and manganese ions had not been discussed (Janusz *et al.* 2015; An *et al.* 2016a). Copper ions were the best inducer among tested metal ions for laccase secretion from *P. ostreatus* and *F. velutipes*.

CONCLUSIONS

1. This study indicated that the biosynthetic potential of white rot fungi could be highly dependent on the type of fungi, as well as the kind and mixed type of metal ion.
2. *Pleurotus ostreatus* CCEF 89 and *Flammulina velutipes* CCMSSC 00114 showed exceptional potential for laccase synthesis through conventional submerged fermentation with copper ions, manganese ions, or a mixture of copper and manganese ions as the inducer.
3. The combination of copper and manganese ions as the inducer for enhancing the laccase activity of *P. ostreatus* was shown to be superior to that of single copper ions or manganese ions as inducers. The single copper ion as inducer for enhancing laccase activity was more suitable for *F. velutipes*, and a mixture of copper and manganese ions was also advantageous for laccase secretion by *F. velutipes.*
4. The maximum laccase activity from *P. ostreatus* CCEF 89 in BM medium, MM 1, MM 2, MM 3, MM 4, MM 5, MM 6, and MM 7 was approximately 21.5-fold, 4.7-fold, 14.9-fold, 16.9-fold, 4.0-fold, 11.0-fold, 12.7-fold, and 24.8-fold higher than that from *F. velutipes* CCMSSC 00114, respectively. The time of maximum laccase activity from *P. ostreatus* occurred later than that in *F. velutipes*.

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