Laccase Produced by *Coriolopsis trogii* and *Cerrena unicolor* with the Mixed of Metal Ions and Lignocellulosic Materials

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Coriolopsis trogii and *Cerrena unicolor* were investigated for laccase production in submerged fermentation with different nutrient medium containing metal ions and lignocellulosic materials. The maximum laccase activity of *C. trogii* Han751 was 8584.44 ± 98.45 U/L and was obtained from nutrient medium 7. However, the maximum laccase activity of *C. unicolor* Han 849 was 16144.26 ± 635.30 U/L from nutrient medium 9. Thus, the capacity of secreting laccase of *C. unicolor* Han 849 was superior to that of *C. trogii* Han751. Different fungal species have different medium components suitable for laccase production. The content of $CuSO_4 \cdot 5H_2O$ and $MnSO_4$ in nutrient medium with the concentration of 0.25 g/L and 0.151 g/L, respectively, was more beneficial to *C. trogii* Han751 secreting laccase. However, the vital components of nutrient medium that contribute to the laccase activity of *C. unicolor* Han 849 were corncob, glucose, and $CuSO_4 \cdot 5H_2O$, with the corresponding concentrations of 1 g/flask, 5 g/L, and 0.25 g/L, respectively. The results will contribute to the development of new methods to produce low-cost laccase.

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Keywords: Coriolopsis trogii; *Cerrena unicolor*; *Laccase*; *Populus beijingensis*; *Corncob*; *Metal ions*

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INTRODUCTION

There is a great need for enzymes for use in biodegradation and biochemical processes (Kremer *et al*. 2015; Rao *et al*. 2019; Han *et al*. 2021b, 2022). Laccase (EC 1.10.3.2), belonging to the blue copper oxidase family; it oxidizes a wide range of phenols and aromatic amines, such as monophenol, diphenol, polyphenol, aminophenol, and methoxyphenol (Gupta and Jana 2019). Laccase was first discovered by Yoshida (1883) in lacquer trees (*Rhus vernicifera*). Supporting studies that identified fungal extracellular secretions as laccase were carried out by Bertrand (1896) and Laborde (1896). Laccase has been found in higher plants, insects, fungi, and bacteria (Geng *et al*. 2018; Unuofin *et al*. 2019b; An *et al*. 2021a, 2021b; Khatami *et al*. 2022). Due to the specificity of low substrate and high catalytic efficiency, laccases have broad application prospects in bio-pulping, biocatalysis, bioremediation, biopolymers, food and beverage processing, biosensor, lignin degradation, nanobiotechnology, and organic synthesis (Upadhyay *et al*. 2016; Bertrand *et*

al. 2017; Hadibarata *et al*. 2018; Yashas *et al*. 2018; Navas *et al*. 2019; Singh and Arya 2019; Unuofin *et al*. 2019a; Zerva *et al*. 2019; Khatami *et al*. 2022; Zhang *et al*. 2022).

Because of the huge application potential of laccase, the search for environmentally friendly and economically feasible compounds to stimulate laccase production has been extensive (Chenthamarakshan *et al*. 2017). The wide use of inexpensive raw materials (industrial and lignocellulosic wastes) containing cellulose, hemicellulose, and lignin to produce laccase can be an economical approach (Leite *et al*. 2019). When lignocellulosic materials are used as nutrients, solid-state fermentation (SSF) is usually adopted. Solid-state fermentation imitates the environment where white-rot fungi grow in nature, and lignocellulosic materials can be used as the substrates for white-rot fungi (WRF) to attach to facilitate their growth (Jaramillo *et al*. 2017). However, solid-state fermentation does not take advantage of industrial production, mainly because it is inconvenient to operate. Therefore, submerged fermentation (SF) is still the main way of industrial production of laccase. Research on WRF laccase production has focused on continuous soaking fermentation using soluble nutrients in complex and synthetic culture medium.

Metal ions, fungal species, particle size of lignocellulosic materials, pH, and temperature all affect the ability of fungi to produce laccase (Atila *et al*. 2017; Kostadinova *et al*. 2018; Gaikwad and Meshram 2019; Jasinska *et al*. 2019; Lallawmsanga *et al*. 2019; Sun *et al*. 2021). Along with the in-depth study of the existing laccase-producing strains, many researchers are also working on the discovery of new laccase-producing fungi (Myasoedova *et al*. 2017; Yadav and Vivekanand 2019). *Coriolopsis trogii* and *Cerrena unicolor* are the model white-rot fungi that produce extracellular enzymes, including laccase. Previous studies have shown that these two fungi have excellent ability of secreting laccase (Han *et al*. 2021b; Qiu and Liu 2022). The appropriate concentration of metal ions (*e.g*., Cd^{2+}, Cu^{2+} and Mn^{2+}) can promote the production of laccase by white-rot fungi, such as *Flammulina velutipes*, *Phlebia radiata*, and *Pleurotus ostreatus*. (Baldrian and Gabriel 2002; Liu *et al.* 2009; Mäkela *et al.* 2013; Janusz *et al*. 2015; An *et al.* 2016, 2020; Zhou *et al*. 2017). The laccase activities of *Pleurotus ostreatus* and *Flammulina velutipes* induced by different metal ions were analyzed by An *et al*. (2020), and the results showed that single copper ion or manganese ion could enhance the laccase activities secreted by *P. ostreatus* and *F. velutipes*. Some types and high concentration of metal ions (*e.g*., Fe^{2+} and Hg^{2+}) which act as an inducer can also inhibit fungal laccase activity (Lorenzo *et al*. 2005; Juarez-Gomez *et al*. 2018; Xu *et al*. 2018; Li *et al*. 2022). Other studies have analyzed the laccase activity of *Coriolopsis trogii* and *Cerrena unicolor* on lignocellulosic materials, such as *Populus beijingensis*, rice straw, cottonseed hull, and corncob (Han *et al*. 2021b, 2022, 2023; Liu *et al*. 2022). The content of lignin, hemicellulose and cellulose of hardwood stems and corncobs was 18-25%, 24-40%, 40-55% and 15%, 35%, 45% (Howard *et al*. 2003; Sanchez 2009). The effects of mixed metal ions, such as Fe^{2+} and Cu^{2+}, on the activity of fungal laccase have been reported (Zhuo *et al*. 2017a,b; An *et al.* 2020). Xu *et al*. (2020) investigated the growth, quality, and ligninolytic enzymes activities of *Lentinula edodes* when fermented on mixed lignocellulosic biomass. Han *et al*. (2021b) analyzed the laccase activity secreted by *Cerrena unicolor* on the mixture of *Pinus tabuliformis* and *Firmiana platanifolia*. However, there are few studies on laccase production by fungi stimulated by mixed conditions of lignocellulosic materials and metal ions. This study examined the laccase activity of *Cerrena unicolor* and *Coriolopsis trogii* under the mixed condition of metal ions and lignocellulosic materials. The results will contribute to developing new low-cost methods to produce laccase.

EXPERIMENTAL

Materials

Microorganisms

Coriolopsis trogii Han751 and *Cerrena unicolor* Han 849 were collected from Maojingba National Nature Reserve (Longhua County, Chengde City, Hebei Province, China) and Wulingshan National Nature Reserve (Xinglong County, Chengde City, Hebei Province, China). These two fungi strains were maintained on malt extract agar (MEA) medium (g/L: glucose 10, malt extract 20, KH_2PO_4 3, and agar 20), and stored at 4 ℃.

Chemicals

2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was 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). Other chemicals used in present work were purchased from Tianjin Zhiyuan Chemical Reagent Co. Ltd. (Tianjin, China).

Lignocellulosic biomass

Corncob was kindly provided by farmers in Chengde city, Hebei province (China), and *Populus beijingensis* was obtained from Langfang city, Hebei province (China). All lignocellulosic biomass was air-dried, then ground by a micro plant grinding machine with the practical size between 20- and 60-mesh.

Methods

Microbial culture

Coriolopsis trogii Han751 and *Cerrena unicolor* Han 849 were isolated from the natural habitat and stored in College of Life Science, Langfang Normal University. All of these fungal strains were cultured on CYM (g/L: glucose 20, peptone 2, yeast extract 2, $MgSO_4 \cdot 7H_2O$ 0.5, $K_2HPO_4 \cdot 3H_2O$ 1, KH_2PO_4 0.46, and agar 15) to perform the activation process of fungi at 26 ℃ for 7 days. Five inoculants with a diameter of 1.0 cm were picked with a round punch tool and inoculated into liquid CYM medium (100 mL) in triangular flask (250 mL). Then, all flasks were transferred to a shaker and cultured at 150 rpm to obtain fungal seed liquid at 26 ℃.

Inoculum preparation

After 7 days, the preparation of the inoculum was obtained by breaking up the mycelium pellets in the triangular flask with a homogenizer at 5000 rpm for 90 s.

Process of submerged fermentation

Triangular flasks (250 mL) containing nutrient medium for inducing laccase production (Table 1) were autoclaved at 121 ℃ for 30 min. After cooling to room temperature, 3 mL of homogenized inoculum was added to each flask. All flasks were transferred to a rotary shaker to perform the submerged fermentation process with the speed of 150 rpm at 26 ℃. The crude enzyme was filtered through a filter paper, then centrifuged at 12000 rpm for 20 min with the temperature of 4 ℃.

Table 1. Description of the Nutrient Medium

Nutrient Medium	Medium Component							
	Corncob (g/flask)	*Populus beijingensis* (g/flask)	Glucose (g/L)	Yeast extract (g/L)	Sucrose (g/L)	$CuSO_4 \cdot 5H_2O$ (g/L)	$MnSO_4$ (g/L)	Peptone (g/L)
NM 1	2	1	20	4	5	1.25	0.755	4
NM 2	2	2	20	1	5	0.25	0.755	1
NM 3	1	1	5	1	5	0.25	0.151	1
NM 4	1	1	5	4	5	1.25	0.755	1
NM 5	2	1	5	1	20	0.25	0.755	4
NM 6	2	2	5	1	5	1.25	0.151	4
NM 7	1	2	20	4	5	0.25	0.151	4
NM 8	1	1	20	1	20	1.25	0.151	4
NM 9	1	2	5	4	20	0.25	0.755	4
NM 10	1	2	20	1	20	1.25	0.755	1
NM 11	2	2	5	4	20	1.25	0.151	1
NM 12	2	1	20	4	20	0.25	0.151	1

Laccase activity assay

Laccase activity was determined by monitoring the oxidation of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) to ABTS-azine at 415 nm ($\varepsilon_{415} = 3.16 \times 10^4$ 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.

Statistical analysis

All experiments were performed in triplicate. The values were expressed as mean ± standard deviation. In colored graphs, standard deviation was represented as error bars. The graphs were generated by the Origin 2016 (OriginLab Corporation, Northampton, MA, USA). Data were subjected to two-way analysis of variance (ANOVA) by SPSS 22.0 (PROC GLM, Armonk, NY, USA).

RESULTS AND DISCUSSION

Effect of Nutrient Medium on Laccase Activity Secreted by *Coriolopsis trogii* Han751

The laccase secretion capacity of *Coriolopsis trogii* had been demonstrated in previous studies (Yan *et al*. 2014; Campos *et al*. 2016; An *et al*. 2021b; Han *et al*. 2022; Liu *et al*. 2022; Qiu and Liu 2022). Previous studies examined laccase activity in solid or submerged fermentation with lignocellulosic materials. As a complex carbon/nitrogen source and energy source for the growth of white rot fungi, lignocellulosic materials are excellent materials for the production of low-cost laccase (Nawaz *et al*. 2019; Wang *et al*. 2019). In addition, metal ions can induce white rot fungi to produce laccase. Thus, the effect of the mixture of metal ions and lignocellulosic materials on laccase activity secreted by *C. trogii* Han751 was analyzed. The results are shown in Fig. 1 and Table 2.

Comparative analysis of laccase activity from *Coriolopsis trogii* Han 751 in submerged fermentation with lignocellulosic biomass showed that the maximum value of laccase activity was 55.35 ± 0.76 U/L, 134.72 ± 3.55 U/L, 72.03 ± 3.06 U/L, and 80.97 ± 2.53 U/L when in Sorghum straw, *Populus beijingensis*, *Toona sinensis*, and *Salix*

babylonica submerged fermentation (An *et al*. 2021b). Han *et al*. (2022) analyzed the laccase activity of species belonging to the genus of *Coriolopsis* fermented on different lignocellulosic wastes, and maximum laccase activity of *Coriolopsis trogii* Han 1211 was 223.03 ± 11.51 U/L, 118.24 ± 3.48 U/L, 151.50 ± 3.32 U/L, and 75.85 ± 3.62 U/L on cottonseed hull, rice straw, corncob, and *Populus beijingensis*, respectively. Liu *et al*. (2022) investigated the laccase activities of *Coriolopsis trogii* Han 751 on solid-state fermentation, and maximum laccase activity of *Coriolopsis trogii* Han 751 on stalk of *Helianthus annuus* (SOHA), stalk of *Sorghum bicolor* (SOSB), *Pinus tabuliformis*, *Populus beijingensis*, cottonseed hull, and corncob was 122.26 ± 4.57 U/L, 799.03 ± 40.89 U/L, 13.96 ± 0.46 U/L, 18.59 ± 0.92 U/L, 16.58 ± 0 U/L, and 90.92 ± 4.01 U/L. The laccase activity of *C. trogii* Han751 in different nutrient media is shown in Table 2 and Fig. 1.

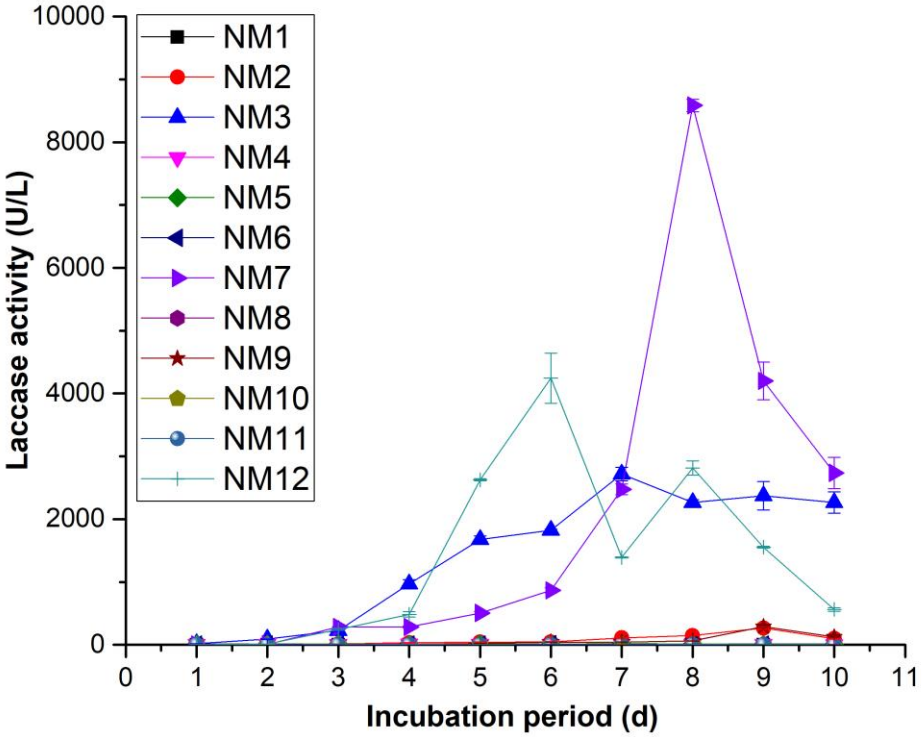

Fig. 1. Laccase activity of *Coriolopsis trogii* Han751 fermented in nutrient medium

Obviously, the laccase activity of *C.* trogii Han 751 fermented with NM7 (containing corncob 1 g/flask, *Populus beijingensis* 2 g/flask, glucose 20 g/L, yeast extract 4 g/L, sucrose 5 g/L, $CuSO_4 \cdot 5H_2O$ 0.25 g/L, $MnSO_4$ 0.151 g/L, and peptone 4 g/L) was 3167.69-fold, 32.16-fold, 3.16-fold, 860.16-fold, 547.83-fold, 1113.42-fold, 1369.13-fold, 29.74-fold, 2189.91-fold, 1524.77-fold, and 2.02-fold. In addition, the maximum laccase activity of *C. trogii* Han 751 under NM3 and NM12 culture conditions was also greater than 2700 U/L (Fig. 1). In conclusion, the content of $CuSO_4 \cdot 5H_2O$ and $MnSO_4$ in nutrient medium of 0.25g/L and 0.151g/L, respectively, was more beneficial to *C. trogii* Han 751 secreting laccase.

Table 2. Maximum Laccase Activity and Occurrence Time of *Coriolopsis trogii* Han 751 and *Cerrena unicolor* Han 849 in Nutrient Medium Submerged Fermentation

Fungi	Nutrient Medium (NM)	Maximum Laccase Activity (U/L)	Time (Day)
Coriolopsis trogii Han 751	1	2.71 ± 0	9th
Coriolopsis trogii Han 751	2	266.93 ± 18.01	9th
Coriolopsis trogii Han 751	3	2717.45 ± 106.91	7th
Coriolopsis trogii Han 751	4	9.98 ± 0.50	4th
Coriolopsis trogii Han 751	5	15.67 ± 0.90	7th
Coriolopsis trogii Han 751	6	7.71 ± 0.37	6th
Coriolopsis trogii Han 751	7	8584.44 ± 98.45	8th
Coriolopsis trogii Han 751	8	6.27 ± 0.21	4th
Coriolopsis trogii Han 751	9	288.63 ± 12.76	9th
Coriolopsis trogii Han 751	10	3.92 ± 0.30	5th
Coriolopsis trogii Han 751	11	5.63 ± 0.17	4th
Coriolopsis trogii Han 751	12	4244.12 ± 400.53	6th
Cerrena unicolor Han 849	1	377.74 ± 10.85	9th
Cerrena unicolor Han 849	2	582.48 ± 15.89	8th
Cerrena unicolor Han 849	3	12517.58 ± 488.15	9th
Cerrena unicolor Han 849	4	1366.28 ± 114.10	9th
Cerrena unicolor Han 849	5	754.17 ± 88.87	7th
Cerrena unicolor Han 849	6	1185.45 ± 69.60	10th
Cerrena unicolor Han 849	7	2822.99 ± 75.85	8th
Cerrena unicolor Han 849	8	7.03 ± 0.17	5th
Cerrena unicolor Han 849	9	16144.26 ± 635.30	8th
Cerrena unicolor Han 849	10	8.04 ± 0.46	5th
Cerrena unicolor Han 849	11	34.06 ± 2.17	7th
Cerrena unicolor Han 849	12	5686.16 ± 453.42	9th
Maximum laccase activity are presented as mean value ± standard deviation for biological triplicates.			

Effect of Nutrient Medium on Laccase Activity Secreted by *Cerrena unicolor* Han 849

Previous studies had indicated excellent laccase secretion ability of *Cerrena unicolor* (Jamroz *et al*. 2004; Rola *et al*. 2013; Kachlishvili *et al*. 2014, 2021; Pawlik *et al*. 2021; Zhang *et al*. 2021). There are also many studies on the effect of lignocellulose materials on laccase activity from *C. unicolor*. The study of metal ions on laccase activity of *C. unicolor* mainly focused on the tolerance of purified laccase to metal ions. Under these conditions, the effect of mixing metal ions and lignocellulosic materials on the activity of laccase secreted by *C. unicolor* Han 849 were examined in the present work.

Han *et al*. (2021a) detected the laccase activity of *C. unicolor* Han 849 on different lignocellulosic residues and maximum laccase activity was 295.96 ± 4.85 U/L on *Populus beijingensis*, 625.98 ± 24.08 U/L on *Firmiana platanifolia*, 371.71 ± 5.69 U/L on *Sorghum bicolor*, and 102.17 ± 3.55 U/L on *Oryza sativa*. Another, Han *et al*. (2021b) also found that mixing lignocellulosic materials could be increased the activity of laccase secreted by the white rot fungus *C. unicolor* Han 849, and the laccase activity in submerged fermentation with *Pinus tabuliformis*, *Firmiana platanifolia*, and the mixture of *Pinus tabuliformis* and *Firmiana platanifolia* was 223.53 ± 21.06 U/L, 552.34 ± 49.14 U/L, and 876.23 ± 20.82 U/L. However, laccase activity of *C. unicolor* Han 849 in submerged fermentation with NM 1, NM 2, NM 3, NM 4, NM 5, NM 6, NM 7, NM 8, NM 9, NM 10,

NM 11, and NM 12 was 377.74 ± 10.85 U/L, 582.48 ± 15.89 U/L, 12517.58 ± 488.15 U/L, 1366.28 ± 114.10 U/L, 754.17 ± 88.87 U/L, 1185.45 ± 69.60 U/L, 2822.99 ± 75.85 U/L, 7.03 ± 0.17 U/L, 16144.26 ± 635.30 U/L, 8.04 ± 0.46 U/L, 34.06 ± 2.17 U/L, and 5686.16 ± 453.42 U/L, respectively (Fig. 2, Table 2). Obviously, nutrient medium of NM 9 and NM 3 were more beneficial to increase laccase activity of *C. unicolor*, followed by NM 12 and NM 7. In this study, the laccase activity of *C. unicolor* Han 849 in nutrient medium of NM9 and NM3 conditions was much higher than that of *C. unicolor* in previous studies.

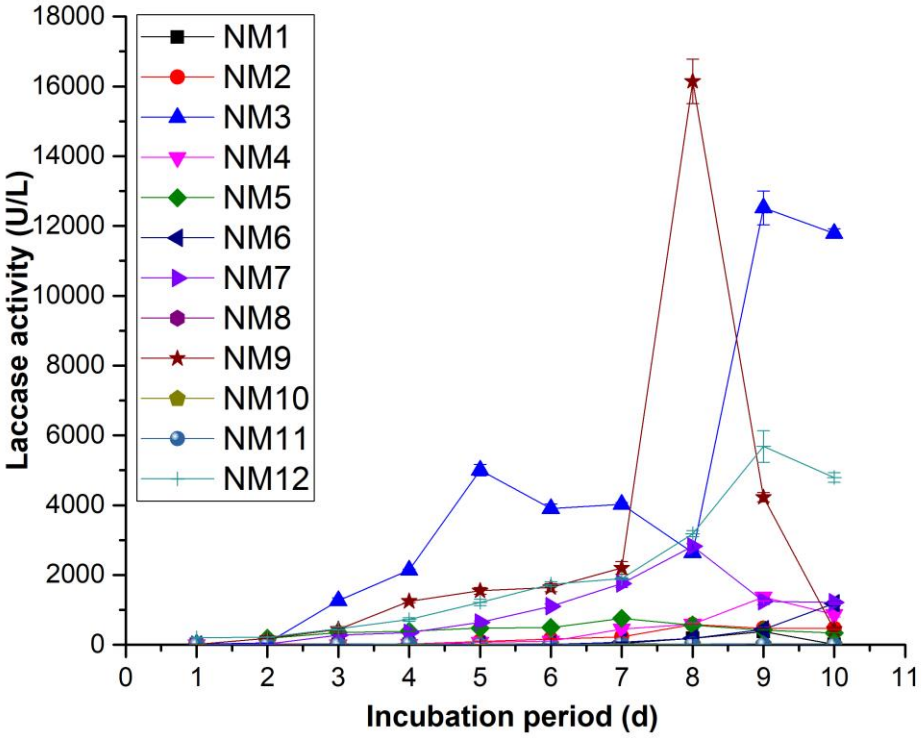

Fig. 2. Laccase activity of *Cerrena unicolor* Han 849 fermented in Nutrient Medium

Comparative Analyze of *Coriolopsis trogii* Han751 and *Cerrena unicolor* Han 849 on Laccase Activity

White-rot fungi, famous for their laccase secreting ability, are widely studied. Meanwhile, the ability of secreting laccase from different species was commonly significant (Elisashvili *et al*. 2008; An *et al*. 2021a; Han *et al*. 2021a). Similarly, laccase activities could be significantly affected by fungal species and nutrient medium ($P < 0.001$).

Maximum laccase activities of *Cerrena unicolor* Han 849 in submerged fermentation of NM 1, NM 2, NM 3, NM 4, NM 5, NM 6, NM 8, NM 9, NM 10, NM 11, and NM 12 was nearly 139.39-fold, 2.18-fold, 4.61-fold, 136.90-fold, 48.13-fold, 153.75-fold, 1.12-fold, 55.93-fold, 2.05-fold, 6.05-fold, and 1.34-fold higher than that of *Coriolopsis trogii* Han751 in corresponding nutrient medium, while maximum laccase activities of *C. trogii* Han751 in NM 7 submerged fermentation was nearly 3.04-fold higher than that of *C. unicolor* Han 849. On the whole, the ability of secreting laccase of *C. unicolor* Han 849 was superior to that of *C. trogii* Han751. Maximum laccase activities of *Trametes trogii* and *T. versicolor* on solid-state fermentation with *Corylus maxima* were

384 U/L and 68 U/L (Birhanli and Yesilada 2013). Gupta and Jana (2019) analyzed the laccase activity of *Ganoderma lucidum* fermented in repeated batch semi-solid fermentation (sSF) process with wheat straw and the optimized highest laccase activity in batch sSF was 15257.2 ± 353.4UL on the 9th day. Previous work (Lorenzo *et al*. 2002) investigated the optimized production of laccase by *T. versicolor* in submerged cultures with the lignocellulosic residues, and the highest activity was 639 U/L with barely bran. Laccase activity obtained in barley bran submerged cultures of *T. versicolor* mixed with Mn^{2+} (1 mM) or Cu^{2+} (2 mM) was 938 U/L or 6342 U/L (Lorenzo *et al*. 2006). Obviously, it can be found that the maximum laccase activity of *C. trogii* Han751 and *C. unicolor* Han 849 in the present study under the condition of nutrient medium 7 (8584.44 ± 98.45 U/L) and nutrient medium 9 (16144.26 ± 635.30 U/L) was higher than the results of some previous studies. However, the maximum laccase activity detected in present study was lower than that from *T. trogii* S0301 (352.09 U/L) after the laccase purification process (ammonium sulfate precipitation, anionic exchange chromatography, and Sephadex G-75 chromatography) (Yan *et al*. 2014).

CONCLUSIONS

1. Overall, the laccase secretion capacity of *Cerrena unicolor* Han 849 in submerged fermentation with nutrient medium was superior to that of *Coriolopsis trogii* Han751.
2. Maximum laccase activity of *Coriolopsis trogii* Han751 obtained from nutrient medium 7 was 8584.44 ± 98.45 U/L, while the maximum laccase activity of *Cerrena unicolor* Han 849 was 16144.26 ± 635.30 U/L from nutrient medium 9.
3. The content of $CuSO_4 \cdot 5H_2O$ and $MnSO_4$ in nutrient medium of 0.25g/L and 0.151g/L, respectively, was more beneficial to *Coriolopsis trogii* Han751 secreting laccase.
4. The vital components of nutrient medium that contribute to the laccase activity of *Cerrena unicolor* Han 849 were corncob, glucose, and $CuSO_4 \cdot 5H_2O$, and corresponding content was 1 g/flask, 5 g/L, and 0.25 g/L.

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