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Original Article

Enhancement of Laccase Production from Wood-Rotting Fungus by Co-Culture with *Trichoderma longibrachiatum*[1]

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ABSTRACT

This work aimed to evaluate the influence of culture conditions on laccase production in the co-culture of wood-rotting fungus with *Trichoderma* sp. The effects of infection extent, infection time, and culture filtrate of *Trichoderma* sp. on the laccase production by wood-rotting fungus in co-culture were examined. *T. rubrum* LKY-7 and *T. longibrachiatum* were selected as fungi which are effective in co-culture for laccase production. A significant increase in laccase activity was observed when *T. rubrum* LKY-7 was co-cultured with *T. longibrachiatum* in glucose-peptone liquid medium, yielding an increase of more than 5 times in laccase activity, as compared with control. Laccase production by *T. rubrum* LKY-7 during co-culturing was significantly influenced by the infection extent and the infection time of *T. longibrachiatum*. Maximal laccase activity was obtained when *T. rubrum* LKY-7 culture was infected by *T. longibrachiatum* after 3 days of cultivation at an inoculum size ratio of 0.5 to 1. The addition of culture filtrate or autoclaved mycelium of *T. longibrachiatum* to *T. rubrum* LKY-7 culture did not significantly enhance laccase production by *T. rubrum* LKY-7 as compared with control (mono cultures of *T. rubrum* LKY-7).

Keywords: laccase, co-culture, infection extent, infection time, culture filtrate

1. INTRODUCTION

Laccase is a blue multi copper-containing oxidase that has been found in numerous basidiomycete fungi, in various plants and insects, and even in bacteria. It has been known that laccase has many physiological functions, including lignin biosynthesis/biodegradation, stress defense, and plant pathogenesis etc. (Thurston, 1994; Cho *et al*., 2012; Lee et al., 2016; An *et al*., 2018). And due to the wide range of substrates, laccase has been shown effective in various biotechnological processes, such as pulping and bleaching, dye decolorization, wastewater treatment, food processing, and bioremediation of environmental pollutants (Choi *et al*., 2006; Hong *et al*., 2013; Pooja *et al*., 2016;). Especially, the fact that laccase require only oxygen for catalysis has attracted much research attention for the practical use of fungal laccase in industry, unlike peroxidase. However, as the extracellular fungal laccase is produced relatively in small amounts, the use of laccase for industrial applications has been limited by low productivities in the processes (Minussi *et al*., 2002).

Laccase production can be enhanced by the addition of various inductive substances, including aromatic or

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phenolic compounds (Valle *et al*., 2015; Yadav, 2018) and metal ions (Baldrian and Gabriel, 2002; Jarosz-Wilkolazka *et al*., 2004) etc. However, most of the chemical laccase inducers are environmentally toxic and expensive. Thus, many researchers have been trying to find the ways, which are safe environmentally and low-cost, to increase laccase production from white-rot fungi.

Interspecific interaction between white-rot fungi and other fungi or bacteria have shown to induce laccase activity (Savoie and Mata, 1999; Baldrian, 2004; Zhang *et al*., 2006; Hiscox, 2010). In interspecific fungal interaction, the extent of the increase in laccase activity could be differ, depending on the innate laccase producing ability of a species, the combination of species interacting (Iakovlev and Stenlid, 2000; Chi *et al*., 2007), and the types of ligninolytic enzyme secreted by each strain (Lee *et al*., 2017). And for high volumetric production of laccase in interspecific fungal interaction, the techniques of submerged fermentation should be applied rather than solid media. However, in submerged cultures, it is difficult to make close contact between strains in comparison with solid cultures. Flores *et al.* (2010) reported that the relative increase in laccase volumetric activity during *Trichoderma-Pleurotus* co-culturing in liquid media was half of that obtained when both strains were directly confronted in solid media, and laccase production in liquid media was influenced by the time of infection and agitation/oxygenation conditions.

In previous work, we evaluated the effect of ligninolytic enzyme type secreted by each strain on ligninolytic enzyme production in interspecific interactions of wood-rotting fungi. In glucose-peptone agar medium, co-cultures of wood-rotting fungi with *Trichoderma viride* which do not produce any ligninolytic enzyme were very effective in the increase of laccase activity (Lee *et al*., 2017). The present work was to select the effective wood-rotting fungus and *Trichoderma* sp. on laccase production in interspecific interaction, then to evaluate the effect of infection extent (inoculum size), infection time, and culture filtrate of *Trichoderma* sp. on laccase production by wood-rotting fungi in submerged co-culture.

2. MATERIALS and METHODS

2.1. Fungal strains and culture conditions

Trychophyton rubrum LKY-7 (*T. rubrum* LKY-7) were isolated from hardwood chip pile (Jung *et al*., 2001), and *Pycnoporus cinnabarinus* (*P. cinnabarinus*) were kindly supplied by Prof. Kaichang Li (Oregon State University, USA). *Trichoderma harzianum*, *Trichoderma longibrachiatum*, *Trichoderma reesei*, and *Trichoderma viride* were obtained from KCCM (Korea Culture Center of Microorganisms). These fungi were maintained on potato dextrose agar (PDA) and stored at 4℃. For screening the effective *Trichoderma* sp. on laccase production by wood-rotting fungi, interspecific interactions were performed on glucose-peptone agar medium (GPA, solid medium) (30 g glucose; 10 g Bacto-peptone; 1.5 g KH_2PO_4; 0.5 g $MgSO_4$; 20 mg $CuSO_4$; 12 mg $MnSO_4$; 2 mg thiamine-HCl; Agar 20 g, per liter) at pH 5. Glucose-peptone broth medium (GPB, liquid medium) containing several glass beads (Ø 0.5 mm) to avoid the formation of mycelial pellet was used for laccase production.

2.2. Selection of effective fungi in laccase production

In order to select effective fungi on laccase production in interspecific interaction, *P. cinnabarinus or T. rubrum* LKY-7 and each *Trichoderma* sp. were inoculated onto cellophane membranes (GelAir Cellophane, Bio-Rad 165-1779) overlaid on GPA plate at a 5 cm apart. The cellophane membrane was boiled twice for 15 min in distilled water, immersed in a flask containing distilled

water, autoclaved for 15 min, and then overlaid on GPA surface (Téllez-Téllez *et al*., 2003). As the growth of each *Trichoderma* sp. was faster than that of wood-rotting fungi, *P. cinnabarinus* and *T. rubrum* LKY-7 were inoculated first, and *Trichoderma* strains were inoculated to those cultures 3 days later, respectively. One mycelial plug (mycelium of 8 mm diameter) was aseptically scraped from the contact zone of *T. rubrum* LKY-7 or *P. cinnabarinus* region in the co-cultures at days 5 and 12 of cultivation after initial contact. Laccase activities were determined from the harvested mycelium according to a previously described method (Lee *et al*., 2017). The self-paired culture of each wood-rotting fungus was used as control.

2.3. Laccase production in submerged co-culture

GPB was used for laccase production by interspecific interaction. Wood-rotting fungus and *Trichoderma* sp. selected from above experiments were pre-cultivated on cellophane membrane overlaid on GPA plate for 7 and 3 days, respectively. The mycelium of 6-10 mm diameter was scraped from actively growing mycelial edge on the surface of cellophane membrane and used for inoculum of each strain. Basically, first, the inoculum of wood-rotting fungus was homogenized with portable mill in 25 ml of GPB and incubated for 3 days at 27℃ with agitation at rate of 150 rpm. Then, the mycelium scraped from *Trichoderma* sp. (non-sporulating mycelium, 3 days old) was homogenized in 25 ml GPB, and added into the 3 days old culture of wood-rotting fungus.

In order to evaluate the effect of infection extent of *Trichoderma* sp. on laccase production by wood-rotting fungus, the inoculum size ratio of *Trichoderma* sp. to wood-rotting fungus was adjusted to 0.25 to 1, 0.5 to 1, 1 to 1, and 2 to 1 using cork borers, then co-cultivations were carried out according to the above procedure. Also, to assess the effect of infection time, the homogenized inoculums of *Trichoderma* sp. with inoculum size ratio of 0.5 to 1 and 1 to 1 were added into wood-rotting fungus cultures incubated for 1, 3, and 5 days, respectively. And, to estimate the effect of culture filtrate of *Trichoderma* sp., only *Trichoderma* sp. which homogenized in 25 ml of GPB with inoculum size ratio of 0.5 to 1 were incubated for 4 days. Then, these cultures were separated with mycelium and culture filtrate through a sterile 0.2 um Millipore filter. The separated mycelium was washed thoroughly with distilled water, homogenized in 25 ml of GPB, and autoclaved at 121℃ for 20 min. The autoclaved mycelium and the culture filtrate were each added into 3 days old cultures of wood-rotting fungus.

All cultures described above were incubated under shaking condition at a rate of 150 rpm at 27℃ for 15 days. For the assay of laccase activity, 200 μl culture fluid was extracted periodically in all cultures and replaced by 200 μl flesh culture medium. The mono-culture of wood-rotting fungus was used as control. All experiments were performed in triplicate.

2.4. Measurement of laccase activity

Laccase activity was determined spectrophotometrically by measuring the oxidation of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonnic acid) (ABTS) in sodium tartrate buffer (50 mM, pH 4.5) at 420 nm (ε max = 3.6×10^4 $M^{-1}cm^{-1}$). One unit (U) of laccase activity was defined as the amount of enzyme catalyzing the production of 1 μmol oxidized product per ml of enzyme solution per minute under the assay conditions (Eggert *et al*., 1996).

2.5. Native polyacrylamide gel electrophoresis (PAGE)

For native PAGE, extracellular enzyme extracts were obtained from mycelia (solid media, GPA) and culture fluids (liquid media, GPB) after 12 days of cultivation, respectively. The scraped mycelia from self-paired

culture and co-culture on GPA were transferred into a 1.5 ml Eppendorf tube, to which 0.5 ml of 50mM sodium tartrate buffer (pH 4.5) was added. Tubes were shaken gently and centrifuged. The filtrates were used as extracellular enzyme samples. And the enzyme extracts in liquid media (GPB) were prepared by centrifugation and filteration (0.2 um Millipore filter) after filtering the culture.

Native PAGE was performed on 10% Mini-PROTEIN TGX gel (BioRad, USA) with running conditions of 35 mA at a constant voltage of 200 V. The amounts of enzyme extract were adjusted to load approximately equivalent laccase activities in each well. For laccase isozyme staining, gel was immersed in 50 mM sodium tartrate buffer (pH 4.5) containing 5 mM guaiacol, and incubated at room temperature until laccase activity bands appeared.

3. RESULTS and DISCUSSION

3.1. Selection of effective fungi on laccase production

In previous work, it showed that laccase production was significantly enhanced in the co-cultures of wood-rotting fungi with *T. viride* which doesn't secrete laccase on GPA when compared with other fungi which secrete laccase (Lee *et al*., 2017). No laccase activities were detected in mono-cultures of four *Trichoderma* strains used in this work (results not shown). Screening strategy for biological induction of laccase production has been generally based in the confrontation of basidiomycetes with other strains and measuring laccase activity in interaction zone (Iakovlev and Stenlid, 2000; Baldrian, 2004; Chi *et al*., 2007). These four strains of *Trichoderma* were subjected to interspecific interaction with *P. cinnabarinus* or *T. rubrum* LKY-7 on cellophane membrane of GPA plate. As shown in Table 1, significant increases of laccase activity were observed when wood-rotting fungi were confronted with *Trichoderma* strains. The highest activity of laccase was obtained when *T. rubrum* LKY-7 was co-cultured with *T. longibrachiatum* on GPA, yielding more than about 8 times increase in laccase activity, as compared with control. It has well known that laccase production of white-rot fungi is effectively enhanced by co-culturing with *Trichoderma* sp. (Baldrian, 2004; Zhang *et al*., 2006; Velázquez-Cedeño *et al*., 2007; Flores *et al*., 2009; 2010). However, the levels of activity in these studies were relatively low when compared with the results of this experiment, although there are large increases in the laccase activities in interspecific interaction.

In self-paired culture (control) of *P. cinnabarinus* or *T. rubrum* LKY-7, the laccase activity at day 5 of

Table 1. Laccase activities of wood-rotting fungi during co-culturing with *Trichoderma* strains in GPA

Strain	Laccase activity (U/ml)[a]			
	P. cinnabarinus		*T. rubrum* LKY-7	
	5 days[b]	12 days	5 days	12 days
Control[c]	0.98±0.23	0.17±0.06	1.45±0.24	0.75±0.17
T. harzianum	1.67±0.21	1.79±0.39	2.48±0.36	2.25±0.49
T. longibrachiatum	3.26±0.47	5.02±0.43	5.32±0.72	8.02±0.75
T. reesi	2.15±0.26	2.42±0.62	2.77±0.49	3.31±0.28
T. viride	5.18±0.74	5.31±0.66	4.19±0.32	4.76±0.69

[a] Laccase activity per ml of enzyme solution extracted from the ground mycelium in 50 mM sodium tartrate buffer
[b] Incubation day after initial contact
[c] Self-paired culture of *P. cinnabarinus* or *T. rubrum* LKY-7

cultivation decreased significantly at day 12. However, in the co-cultures, the stability of laccase activities were improved with compared to control, rather showing the increase of laccase activities at day 12 of cultivation. Especially, in the co-culture of *T. rubrum* LKY-7 with *T. longibrachiatum*, the maximal laccase activity was remained almost constant until day 15 of cultivation (data not shown). These results are in agreement with the study of Flores *et al*. (2010), who has shown that the stability of laccase activity is improved during *Trichoderma-Pleurotus* interaction, yielding twice the activity if compared with control treatments (*Trichoderma* or *Pleurotus* alone). Also, Zhang *et al*. (2006) reported that the laccase activity decreases sharply in chemical induction within 2-3 days after peak, whereas it maintains 60-70% of its highest activity for at least 20 days during the co-cultivation process of *Trametes* sp. and *Trichoderma* strain. Based on these results, it was confirmed that the co-culture of *T. rubrum* LKY-7 with *T. longibrachiatum* has a great potential for laccase production.

3.2. Effect of the infection extent of *T. longibrachiatum* on laccase production

In submerged co-culture, laccase production could be influenced by the inoculum amount of each strain, unlike solid culture. The mycelial suspensions of *T. longibrachiatum* adjusted the inoculum size ratio were added into 3 days old cultures of *T. rubrum* LKY-7 to evaluate the effect of infection extent on laccase production. As shown in Fig. 1, *T. rubrum* LKY-7 produced relatively high activity of laccase when cultivated on GPB (control), and its maximum activity maintained almost the same until the end of cultivation, unlike on GPA. The laccase productions in co-cultures were clearly divided into two levels according to the inoculum size ratio. When the inoculum size ratio of *Trichoderma* sp. to *T. rubrum* LKY-7 were adjusted

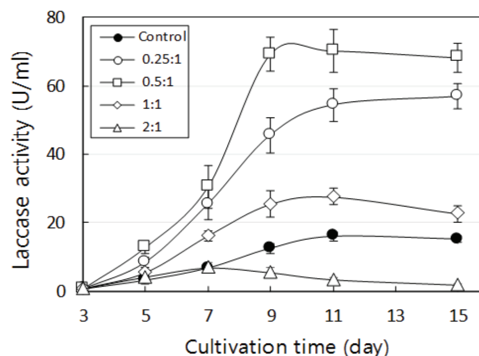

Fig. 1. Effect of the infection extent of *T. longibrachiatum* on laccase production by *T. rubrum* LKY-7.

to 0.25 to 1 or 0.5 to 1, the laccase activities began to increase on days 5 of cultivation, then increased to 54.3 and 70.2 U/ml on days 11, respectively. These laccase activities remained relatively stable until the end of cultivation. It suggested that the laccase activity can be induced strongly when the growth *T. rubrum* LKY-7 has a predominance in co-culture (ratio of 0.25 to 1 or 0.5 to 1). In contrast, when adjusted the inoculum size ratio to 1 to 1, the laccase activities of *T. rubrum* LKY-7 significantly decreased, in comparison with that of a ratio of 0.25 to 1 or 0.5 to 1. Especially, at the ratio of 2 to 1, no induction effect on the laccase activity was observed, which indicated that when the infection extent of *T. longibrachiatum* is beyond a certain amount so that its growth is overgrown in co-culture, the laccase production by *T. rubrum* LKY-7 could be suppressed and inhibited. These results are in agreement with Velázquez-Cedeño *et al*. (2007), who reported that the laccase production in interspecific interaction of *Pleurotus ostreatus* with *T. longibrachiatum* seems to depend on the relative ability of the two fungi to colonize liquid or solid medium. Cupul *et al*. (2014) reported that the low inoculum amount enhances laccase activity by *T. maxima* in *T. maxima-P. carneus* co-cultures, and the amount of mycelial disks used can affect enzyme activity in co-cultures.

On the whole, the inoculations in submerged culture are performed with the addition of homogenized mycelium, fungal agar plug covered with mycelium, and spore suspension (Baldrian, 2004; Mata *et al*., 2005; Flores *et al*., 2010). However, these ways are difficult to inoculate a certain amount of mycelium. Likewise, the inoculum size ratios in this experiment do not represent the exact mycelium amount inoculated to culture, because the mycelium density of each strain is different on GPA. But the results from this work clearly showed that the inoculum amount ratio of two strains plays an important role in laccase production during co-culturing.

3.3. Effect of the infection time of *T. longibrachiatum* on laccase production

The effects of infection time of *T. longibrachiatum* to *T. rubrum* LKY-7 culture on laccase production at the inoculum size ratio of 0.5 to 1 and 1 to 1 were evaluated. As shown in Fig. 2A, when infected with *T. longibrachiatum* after 1 day of cultivation at the ratio of 0.5 to 1, the laccase productions by *T. rubrum* LKY-7 were significantly suppressed and limited. Maximal laccase activity was achieved from the *T. rubrum* LKY-7 culture infected after 3 days at the ratio of 0.5:1, yielding 70.2 U/ml of laccase activity on day 11 of cultivation. And, although the laccase activity increased to some extent when infected after 5 days, it was not as high as when infected after 3 days. In contrast, the laccase activities of *T. rubrum* LKY-7 were almost inhibited when infected after 1 day at the ratio of 1 to 1 (Fig. 2B). However, the laccase activity of culture when infected after 5 days at the ratio of 1 to 1 increased gradually until day 7 of cultivation, then increased rapidly until the end of cultivation. And the laccase activities of *T. rubrum* LKY-7 culture when infected after 5 days cultivation were higher than those of the culture infected after 3 days cultivation at the ratio of 1 to 1. These results showed the importance of the growth balance for laccase production in co-culture. That is, it means that for the enhancement of laccase production, *T. rubrum* LKY-7 should be pre-cultured enough to gain a competitive advantage in co-culture, as the infection extent of *T. longibrachiatum* is high (the ratio of 1 to 1). Likewise, Cupul *et al.* (2014) showed that the time at which *T. maxima* is inoculated with *P. carneus* has a significant effect on laccase activity, and the best time for inoculation with *P. carneus* is 3 d after *T. maxima* was established.

The stability of laccase activity is as important as laccase production for industrial and environmental applications. The stability of laccase activity in co-

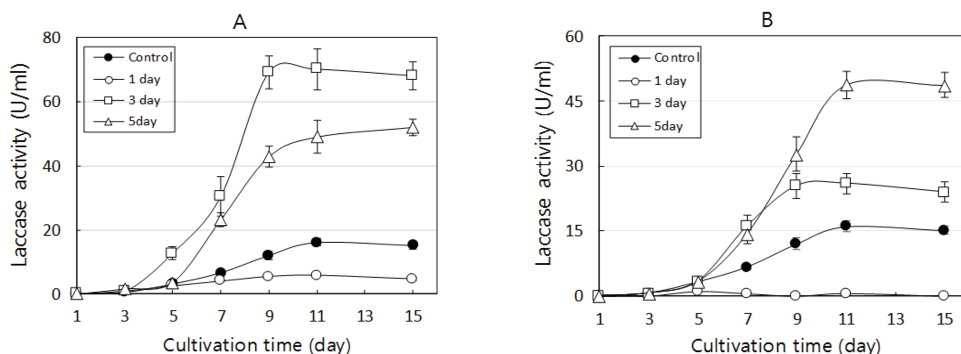

Fig. 2. Effect of the infection time of *T. longibrachiatum* on laccase production by *T. rubrum* LKY-7. A, Inoculum size ratio of 0.5 to 1; B, Inoculum size ratio of 1 to 1.

culture was improved with compared to control, rather showing the increase of laccase activities at day 12 of cultivation. Especially, in the co-culture of *T. rubrum* LKY-7 with *T. longibrachiatum*, the maximal laccase activity was remained almost constant until day 15 of cultivation (data not shown). These results are in agreement with the study of Flores *et al*. (2010), who has shown that the stability of laccase activity is improved during *Trichoderma-Pleurotus* interaction yielding twice the activity if compared with control treatments (*Trichoderma* or *Pleurotus* alone). Also, Zhang *et al*. (2006) reported that the laccase activity decreases sharply in chemical induction within 2-3 days after peak, whereas it maintains 60-70% of its highest activity for at least 20 days during the co-cultivation process of *Trametes* sp. and *Trichoderma* strain.

Generally, it has well known that the increase of laccase activity by basidiomycetes in co-culture could be a response against the attack of *Trichoderma* (Boddy, 2000). However, the results from this experiment showed that the effect of infection extent and infection time on laccase production should be considered in its aspects of the growth ability of *T. longibrachiatum* in co-culture. That is, the increase of laccase activity in co-culture was seemed to be related to the competitive ability of *T. rubrum* LKY-7 against *T. longibrachiatum*. In addition, it suggested that for laccase production, the infection extent and the infection time in co-culture should be adjusted so that *T. rubrum* LKY-7 can be grown up in advance to a certain level, before inoculating *T. longibrachiatum*.

3.4. Effects of the culture filtrate and the autoclaved mycelium of *T. longibrachiatum* on laccase production

The addition of culture filtrate or autoclaved mycelium of *T. longibrachiatum* to *T. rubrum* LKY-7 culture did not significantly enhance laccase production.

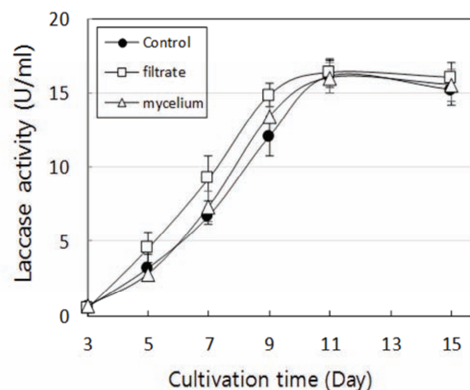

Fig. 3. Effects of the culture filtrate and the autoclaved mycelium of *T. longibrachiatum* on laccase production by *T. rubrum* LKY-7.

As shown in Fig. 3, although the laccase activity of *T. rubrum* LKY-7 culture increased to some extent with the addition of culture filtrate, it was quite a low level, when compared with the maximal laccase activity obtained from co-culture. And the addition of autoclaved mycelium appeared to be little effect on the enhancement of laccase production. This suggested that laccase production in co-culture was enhanced mainly by the live and active mycelium of *T. longibrachiatum*. These results are in agreement with Baldrian (2004), who reported that the addition of sterilized *T. harzianum* culture homogenate did not significantly affect the enzyme activity in the decolorization of RBBR. Also, the same results (no laccase induction) were obtained when sterile-filtered culture liquid from *T. versicolor* x *T. harzianum* cultures were added to the flasks with *T. versicolor*. On the other hand, it has been proposed that *Trichoderma* sp. and/or its metabolites stimulate the laccase production of ligninolytic fungi (Savoie *et al*., 1998; 2001; Savoie and Mata, 1999, Hatvani *et al*., 2002). Mata *et al*. (2005) demonstrated that a significant increase in laccase activity was observed when cultures of *P. pulmonarius* were incubated with the *Trichoderma* lytic enzyme, representing more than an eight-fold increase in production compared to the

P. pulmonarius control. Also, it has been suggested that extracellular laccase production of *L. edodes* may be due to cell wall degrading enzymes produced by *Trichoderma* (Savoie and Mata, 2003). In contrast, Zhang *et al*. (2006) suggested that some thermal-resistant compounds, but not lyases, in the extracellular metabolites of *Trichoderma* sp. ZH1 can singly act as signals to induce laccase production of *Trametes* sp. AH28-2 strongly. This implies that recognition of the host (resulting in a direct attack to basidiomycetes) by *Trichoderma* and its production of non-enzymatic metabolites are both factors worth considering the potential for increased laccase production by *P. ostreatus* or *A. bisporus* (Flores *et al*., 2009). However, the results from this work showed that the live and active mycelium of *T. longibrachiatum* is more effective on the enhancement of laccase production by *T. rubrum* LKY-7 in co-culture than its culture filtrate or auto-claved mycelium.

3.5. Laccase isozymes in native PAGE

The native PAGE showed that *T. rubrum* LKY-7 produced two major bands (band 1 and 2) and two minor bands (band 3 and 4) of laccase activities (Fig. 4). Mata *et al*.(2005) observed two patterns of main bands formed by *Pleurotus* strain according to the kind of culture media with or without *Trichoderma*. Flores *et al*. (2010) reported that *Pleurotus ostreatus* produces two laccase band, which are always present throughout the cultivation time, while laccase isoforms pattern changed after the infection of *P. ostreatus* cultures with *T. viride*. In this work, no inducible laccase isozyme or laccase modification was observed both on GPA and GPB. It showed that when co-cultured with *T. longibrachiatum*, the intensity of laccase band 4 increases both on GPA and GPB in comparison with that of mono-culture of *T. rubrum* LKY-7 on GPB. From these results, it could be supposed that laccase

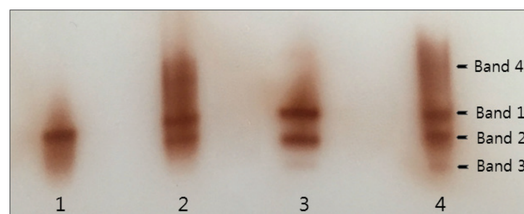

Fig. 4. Detection of laccase isozymes by native PAGE.
Lane 1, self-paired culture of LKY-7 in GPA
Lane 2, co-culture in GPA
Lane 3, mono-culture of LKY-7 in GPB
Lane 4, co-culture in GPB.

band 4 was related to the enhancement of laccase production in co-cultivation on GPA and GPB. Interestingly, only one laccase band was detected in self-paired culture of *T. rubrum* LKY-7 on GPA, while all laccase isoforms were observed in mono-culture of *T. rubrum* LKY-7 on GPB. Mata *et al*. (2005) reported that the presence of *Trichoderma* does not seem to induce new laccase isoforms, but rather, it only seems to stimulate the overproduction of this enzyme. Flores *et al*. (2009) have shown that while two laccases were observed in liquid co-culture, only one isoform was observed during confrontation of *P. ostreatus* with *T. viride* in solid media. And they added that fungal interaction in solid media, although yielding only one laccase, stimulates the production of a highly reactive isoform. Unlike those results, the expression of only one laccase band could be involved in the stability of laccase activity on GPA. That is, the laccase activity decreased significantly at day 12 of cultivation in self-paired culture on GPA (Table 1), at which only one laccase band was detected. On the other hand, the laccase activity remained relatively stable until the end of cultivation in mono-culture in GPB (Fig. 1), at which laccase isoforms pattern did not change. Accordingly, it could be considered that some laccase isozymes in self-paired culture on GPA were removed by autolysis, resulted in the significant decrease of laccase activity at the latter period of cultivation.

4. CONCLUSION

The co-culture of *T. rubrum* LKY-7 with *T. longibrachiatum* was found to have a potential for laccase production and for laccase stability in GPB (liquid medium). A significant increase of laccase activity was observed when *T. rubrum* LKY-7 was co-cultured with *T. longibrachiatum* in GPB, yielding an increase of more than 5 times in laccase activity, as compared with control. The laccase production by *T. rubrum* LKY-7 during co-culturing was greatly influenced by the infection extent and the infection time of *T. longibrachiatum*. It showed that for the enhancement of laccase production, the infection extent and the infection time of *T. longibrachiatum* in co-culture should be adjusted so that *T. rubrum* LKY-7 can grow in advance to a certain level, before inoculating *T. longibrachiatum*. Maximal laccase activity was obtained when the *T. rubrum* LKY-7 culture was infected by *T. longibrachiatum* after 3 days of cultivation at 0.5 to 1 of inoculum size ratio of *T. longibrachiatum* to *T. rubrum* LKY-7 in GPB. The addition of culture filtrate or autoclaved mycelium of *T. longibrachiatum* into *T. rubrum* LKY-7 culture did not significantly enhance the laccase production by *T. rubrum* LKY-7 when compared with control (mono-culture of *T. rubrum* LKY-7). In native PAGE, no inducible laccase isozyme was observed in co-cultures in comparison with the mono-culture of *T. rubrum* LKY-7, only showing the increase of intensity of laccase band both on GPA and GPB. On the other hand, only one laccase band was detected in self-paired culture of *T. rubrum* LKY-7 on GPA, while all laccase isoforms were observed in mono-culture of *T. rubrum* LKY-7 on GPB.

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REFERENCES

An, J.E., Lee, S.Y., Ryu, S.H., Kim, M.K. 2018. Transformation of terpene synthase from *Polyporus brumalis in Pichia pastoris* for recombinant enzyme solution. Journal of the Korean Wood Science and Technology 46(4): 415-422.

Baldrian, P. 2004. Increase of laccase activity during interspecific interaction of white rot fungi. FEMS Microbiology Ecology 50: 245-253.

Baldrian, P., Gabriel, J. 2002. Copper and cadmium increase laccase activity in *Pleurotus ostreatus*. FEMS Microbiology Letters 206: 69-74.

Boddy, L. 2000. Interspecific combative interactions between wood-decaying basidiomycetes. FEMS Microbiology Ecology 31: 185-194.

Chi, Y., Hataka, A., Maijala, P. 2007. Can co-culturing of two white rot fungi increase lignin degradation and the production of lignin degrading enzymes? International Biodeterioration and Biodegradation 59: 32-39.

Cho, M.K., Ryu, S.H., Kim, M.K. 2012. Changes in activities of lignin degrading enzymes and lignin content during degradation of wood chips *by Polyporus brumalis*. Journal of the Korean Wood Science and Technology 40(6): 424-430.

Choi, Y.J., Shin, Y.S., Cho, N.S. 2006. Screening of wood-rot fungi based on RBBR decolorization and its laccase activity. Journal of the Korean Wood Science and Technology 34(4): 46-53.

Cupul, W.C., Abarca, G.H., Carrera, D.M., Vázquez, R.R. 2014. Enhancement of ligninolytic enzyme activities in a *Trametes maxima-Paecilomyces carneus* co-culture : Key factors revealed after screening using a Plackett-Burman experimental design. Eletronic Journal of Biotechnology 17: 13-25.

Eggert, C., Temp, U., Eriksson, K-EL. 1996. The ligninolytic system of the white rot fungus

Pycnoporus cinnabarinus: purificaion and characterization of the laccase. Applied and Environmental Microbiology 62: 1151-1158.

Flores, C., Casasanero, R., Trejo-Hernández, M.R., Galindo, E., Serrano-Caqrreón, L. 2010. Production of laccases by *Pleurotus ostreatus* in submerged fermentation in co-culture with *Trichoderma viride*. Journal of Applied Microbiology 108: 810-817.

Flores, C., Vidal, C., Trejo-Hernández, M.R., Galindo, E., Serrano-Caqrreón, L. 2009. Selection of *Trichoderma* strains capable of increasing laccase production by *Pleurotus ostreatus* and *Agaricus bisporus* in dual cultures. Jounal of Applied Microbiology 106: 249-257.

Hatvani, N., Kredics, L., Antal, Z., Mécs, I. 2002. Changes in activity of extracellular enzymes in dual cultures of *Lentinula edodes* and mycoparasitic *Trichoderma* strains. Journal of Applied Microbiology 92: 415-423.

Hiscox, J., Balddrian, P., Rogers, H.J., Boddy, L. 2010. Changes in oxidative enzyme activity during interspecific mycelial interactions involving the white-rot fungus *Trametes versicolor*. Fungal genetics and Biology 47: 562-571.

Hong, C.H., Kim, H.Y., Jang, S.K., Choi, I.G. 2013. Screening of outstanding white rot fungi for biodegradation of organosolv lignin by decolorization of Remazol Brilliant Blue R and ligninolytic enzymes systems. Journal of the Korean Wood Science and Technology 41(1): 19-32

Iakovlev, A., Stenlid, J. 2000. Spatiotemporal patterns of laccase activity in interacting mycelia of wood-decaying basidiomycete fungi. Microbial Ecology 39: 236-245.

Jarosz-Wilkolazka, A., Malarczyk, E., Leonowicz, A., Cho, N.S. 2004. Effect of cadium ions on the activity of fungal laccase and its decolorization of dye, RBBR. Journal of the Korean Wood Science and Technology 32(6): 14-22.

Jung, H.C., Feng, X, Li., K. 2001. Purification and characterization of laccase from wood-degrading fungus *Trichophyton rubrum* LKY-7. Enzyme and Microbial Technology 30: 161-168.

Lee, K.Y., Park, S.G., Park, I.H., Kim, J.S., Park, M.S., Jung, H.C. 2017. Mycelial response and ligninolytic enzyme production during interspecific interaction among wood-rotting fungi. Journal of Mushrooms 15(4): 168-177.

Lee, S.Y., Ryu, S.H., Choi, I.G., Kim, M.K. 2016. Biosynthesis of Eudesmane-type Sesquiterpenoids by the Wood-rotting Fungus, Polyporus brumalis, on Specific Medium, including Inorganic Magnesium Source 1. Journal of the Korean Wood Science and Technology 44(2): 253-263.

Mata, G., Murrieta Hernández, D.M., Iglesias Andreu, L.G. 2005. Changes in lignocellulolytic enzyme activities in six *Pleurotus* spp. strains cultivated on coffee pulp in confrontation with *Trichoderma* spp. World Journal of Microbiology and Biotechnology 21: 143-150.

Minussi, R.C., Pastore, G.M., Duran, N. 2002. Potential applications of laccase in the food industry. Trends in Food Science and Technology 13: 205-216.

Pooja, U., Rahul, S., Pavan-Kumar, A. 2016. Bioprospecting and biotechnological applications of fungal laccase. 3 Biotech 6: 15-26.

Savoie, J.M., Mata, G. 1999. The antagonistic action of *Trichoderma* sp. hyphae to *Lentinula edodes* hyphae changes lignocellulolytic activities during cultivation in wheat straw. World Journal of Microbiology and Biotechnology 15: 369-373.

Savoie, J.M., Mata, G., Billette, C. 1998. Extracellular laccase production during hyphal interactions between *Trichoderma* sp. and Shiitake, *Lentinula edodes*. Applied Microbiology and Biotechnology 49: 589-593.

Savoie, J.M., Mata, G., Mamoun, M. 2001. Variability in brown line formation and extracellular laccase

production during interaction between white-rot basidiomycetes and *Trichoderma harzianum* biotype Th2. Mycologia 93: 243-248.

Téllez-Téllez, M., Díaz-Godínez, G., Sánchez, C. 2003. Physiology of colony of *Pleurotus pulmonarius* grown on medium overlaid with a cellophane membrane. Applied Microbiology and Biotechnology 63: 212-216.

Thurston, C.F. 1994. The structure and function of fungal laccase. Microbiology 140: 19-26.

Valle, J.S., Vandenberghe, L.P.S., Oliveira1, A.C.C., Tavaresl, M.F., Linde, G.A., Colauto, N.B., Socco, C.R. 2015. Effect of different compounds on the induction of laccase production by *Agaricus blazei.* Genetics and Molecular Research 14: 15882-15891.

Velázquez-Cedeño, M., Farnet, A.M., Billette, C., Mata, G., Savoie, J.M. 2007. Interspecific interactions with *Trichoderma longibrachiatum* induce *Pleurotus ostreatus* defence reactions based on the production of laccase isozymes. Biotechnology Letters 29: 1583-1590.

Yadav, B. 2018. Induction of laccase in fungus, *Cyathus stercoreus* using some aromatic inducers. Journal of Applied and Natural Science 10(1): 445-447.

Zhang, H., Hong, Y.Z., Xiao, Y.Z., Yuan, J., Tu, X.M., Zhang, X.Q. 2006. Efficient production of laccase by *Trametes* sp. AH28-2 in co-cultivation with *Trichoderma* strain. Applied Microbiology and Biotechnology 73: 89-94.