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Research Note

Overproduction of Laccase by the White-Rot Fungus *Pleurotus ostreatus* Using Apple Pomace as Inducer

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Abstract Laccase activity of *Pleurotus ostreatus* is significantly increased by the addition of apple pomace. Among various conditions, the best concentration of apple pomace and cultivation time for the production of laccase by *P. ostreatus* was 2.5% and 9 days, respectively. Reverse transcription polymerase chain reaction analyses of laccase isoenzyme genes, including *pox1*, *pox3*, *pox4*, *poxc*, *poxa3*, and *poxa1b*, revealed a clear effect of apple pomace on transcription induction. Our findings reveal that the use of apple pomace can be a model for the valuable addition of similar wastes and for the development of a solid-state fermenter and commercial production of oyster mushroom *P. ostreatus*.

Keywords Apple pomace, Laccase, *Pleurotus ostreatus*

Pleurotus ostreatus (white-rot fungi), also known as oyster mushroom, is commercially important in the world mushroom market. In addition to its use in food production, *P. ostreatus* has received increasing attention for applications in bio-bleaching and the catalysis of difficult chemical conversions in the paper industry, textile dye decolorization, and detoxification of environmental pollutants [1-4]. Therefore, many efforts are being focused on the molecular characterization of fungal laccases as well as on improving laccase production levels. *Pleurotus ostreatus* excretes the typical laccase isoenzyme POXC [5], the white laccase isoenzyme POXA1w [6], the heterodimeric laccase isoenzyme POXA3 [7-9], and POXA1b [10].

Apple and apple products are among the major fruit and fruit products consumed worldwide. Several million tons of apple pomace are generated during the processing of apple products [11]. Apple pomace is a rich source of nutrients such as carbohydrates, dietary fiber, minerals, and vitamin C [11]. In addition, apple pomace has been used as a raw material in applications such as pectin recovery and the production of enzymes, organic acids, ethanol, and animal feed [10, 12-16]. Because sustainable food production and value addition of wastes are among the most important issues in the agro and food processing industries, the aim of this study was to investigate the effect of apple pomace on laccase production by *P. ostreatus* mycelium.

Pleurotus ostreatus ASI 2344 was obtained from the Korean Agricultural Culture Collection (Suwon, Korea) and cultured on mushroom complete medium (0.45 g KH_2PO_4, 0.5 g $MgSO_4$, 1 g K_2HPO_4, 2 g yeast extract, 2 g bacto peptone, and 20 g glucose per liter) at 25°C. Apple pomace was collected from Chungbuk Wonye Nonghyup, Chung-buk, Korea. The pomace was washed five times with sterilized distilled water to remove any adhering substances, freeze-dried, powdered, and passed through a sieve to obtain uniformly sized particles. The effect of apple pomace on laccase activity was determined by adding pretreated apple pomace to the basal medium (2.5% and 5%; w/v). For laccase enzyme assay and total RNA isolation from mycelia, 2,000 mL flasks containing 500 mL of mushroom complete medium were inoculated (50 mycelial plugs/flask) with fresh plugs from the plate and incubated at 25°C for 20

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Table 1. Primers used for reverse transcription polymerase chain reaction

Gene	Accession No.	Forward (5'-3')	Reverse (5'-3')
β-Actin	Control	tggacaagtcatcaccatcg	gaagcacttgcgatgaacaa
pox1	Genbank Z34847	tcactctttgcaggtcatcg	cgaagtcgtggtagggtcat
pox3	EMBL FM202672	acattggcacggtctctacc	gggtcgcagtcatcgtaaat
pox4	EMBL FM202673	ccggatcaagctggtacatt	gggagtagggattggtccat
poxc	Genbank Z34848	atccagtagttgtcaacggc	cgcttgaggattggtaccat
poxa3	EMBL AJ344434	ggtgttgggtcgtgctctat	gatcccaacgatcctctgaa
poxa1b	Genbank AJ005018	tcaccatccgatttgtagca	taggagtttcgatgggttcg

days with agitation.

Laccase activity was determined via modified oxidation of 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid; ABTS; Sigma, St. Louis, MO, USA) as reported previously [17]. The assay mixture contained 9 μL ABTS (1.8 mM, Sigma) and 10 μL culture supernatant in 181 μL of sodium acetate buffer (50 mM, pH 4.5). Oxidation of ABTS was monitored by determining the increase at 420 nm (ε_{420}, 36,000/M/cm). One unit of laccase activity was defined as the amount of substrate in micromoles transformed per minute, reported in units per volume. All experiments were performed three times by using three replicates for each set of conditions and each time.

Zymograms were used to determine laccase activity with a modified sodium dodecyl sulfate polyacrylamide gel electrophoresis technique [11, 17, 18]. The separating and stacking gels were 12% and 5% acrylamide, respectively, and the electrode reservoir solution was 25 mM Tris, 192 mM glycine, pH 8.4. Gels were stained for laccase activity using 5 mM ABTS as the substrate. Total extracellular protein of the culture supernatant was measured using the Bradford method with bovine serum albumin as the standard.

Total RNA was prepared using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA (10 μg) was further processed with RQ1 RNase-free DNase (Promega, Madison, WI, USA) following the manufacturer's instructions. For reverse transcription polymerase chain reaction (RT-PCR) analysis, the reverse transcription of RNA (1 μg) was performed in a 20-μL reaction volume using oligo-dT18 and ImProm-II reverse transcriptase (Promega) according to the manufacturer's instructions. The PCR reaction was conducted in a 50-μL reaction mixture containing 10 mM deoxyribonucleoside triphosphate mixture, 10 pmol of each specific primer (Table 1), 1 U Taq-polymerase (TaKaRa Korea Biomedical Inc., Seoul, Korea), 10× PCR buffer (100 mM Tris-Cl, pH 8.3, 500 mM KCl, and 25 mM $MgCl_2$), and 1 μL cDNA product. Each reaction included an initial 5 min of denaturation at 94°C, followed by 30 cycles of amplification (94°C for 15 sec, 60°C for 15 sec, and 72°C for 30 sec), and final extension for 5 min at 72°C. Subsequently, 5 μL of each reaction mixture was separated on a 1.8% agarose gel. Primers were designed from the laccase cDNAs *poxc* (GenBank accession No. Z34848), *pox1* (GenBank accession No. Z34847), *poxa1b* (GenBank accession No. AJ005018), *pox3* (EMBL accession No. FM202671), *pox4* (EMBL accession No. FM202672), and *poxa3* (EMBL accession No. AJ344434). The mRNA levels of genes were normalized to the mRNA level of the β-actin gene.

To evaluate the effect of apple pomace on laccase production by *P. ostreatus*, we tested different culture conditions with various concentrations of apple pomace. Laccase activity in the medium with 2.5% (w/v) apple

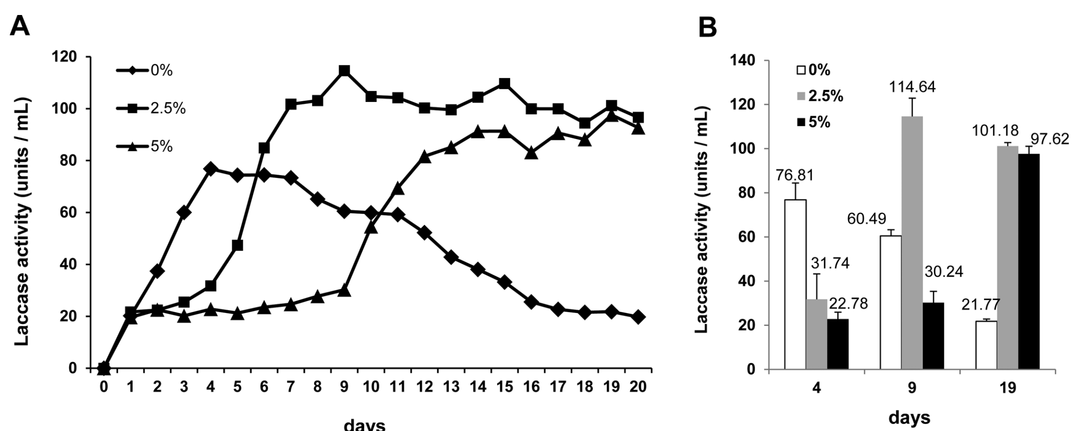

Fig. 1. A, B, Laccase activity (U/mL) of *Pleurotus ostreatus*. Time course of laccase activity in culture of *P. ostreatus* supplemented with different concentrations of apple pomace (0%, 2.5%, and 5%).

pomace drastically increased from days 4 to 9 and showed maximum laccase activity (114.64 U/mL) on day 9 (Fig. 1A). This activity was approximately 280% (30.24 U/mL) and 90% (60.49 U/mL) higher than that of *P. ostreatus* with 5% (w/v) and without apple pomace, respectively (Fig. 1B). This laccase activity was slightly decreased but sustained from days 9 through 20. The laccase activity in the medium without apple pomace was drastically increased from days 1 to 4 and showed maximum laccase activity on day 4 (76.81 U/mL) (Fig. 1). After day 4, laccase activity continuously decreased through day 20 (21.77 U/mL) (Fig. 1B). Interestingly, laccase activity in the medium with 5% apple pomace was slightly increased from days 1 through 9 (30.24 U/mL) but drastically increased from day 9 through day 20 (97.62 U/mL) (Fig. 1A and 1B). The laccase activity in the medium with 2.5% and 5% apple pomace was drastically increased on days 4 and 9, respectively (Fig. 1A). By contrast, the laccase activity in the medium without apple pomace drastically increased from day 1 through day 4. In the same context, the laccase activity in the medium without apple pomace began to decrease on day 4 contrary to laccase activity in the medium with 2.5% apple pomace. Likewise, the laccase activity in the medium with 2.5% apple pomace began to decrease on day 9 contrary to laccase activity in the medium with 5% apple pomace. Interestingly, these obvious laccase activities were observed at nearly 4-day intervals (Fig. 1A). These results suggest that *P. ostreatus* might use the nutrient content of apple pomace without laccase activity in the initial stages of cultivation (approximately 4 days) because apple pomace is rich in carbohydrates, dietary fiber, and minerals [11]. Contrary to expectations, after the addition of two times more apple pomace, the medium with 5% apple pomace showed a laccase activity level similar to that measured after adding 2.5% apple pomace on day 20 (Fig. 1A and 1B). There are several possible explanations for this result. First, considering that lignin metabolism is part of fungal secondary metabolism [19], these results may be related to the fermentation period. Several researchers have reported that high lignin degradation of lignocellulosic substrates using *P. ostreatus* is usually obtained after 60 days of fermentation [15, 19]. Second, the high viscosity of the pomace may adversely affect oxygen dissolution, limiting the growth rate [12]. During the secondary phase of fungal metabolism, lignin degradation is a strictly oxidative process and thus requires oxygen partial pressures similar to the atmospheric value [16]. Third, apple pomace contains several mineral nutrients, including potassium (0.95%), calcium (0.06%), sodium (0.2%), magnesium (0.02%), copper (1.1 mg/L), manganese (8.5~9 mg/L), and iron (230 mg/L) [11]. Copper is reportedly a strong laccase inducer in several species, including *P. ostreatus* [20], *Phanerochaete chrysosporium* [18], and *Trametes versicolor* [21]. Copper induces both laccase transcription and activity [21], and the increase in activity is proportional to the amount of copper added. However, in contrast to other essential metals, copper is toxic to most fungi even at very low concentrations [22]. In addition, Tlecuitl-Beristain *et al.* [17] have reported that high concentrations of copper in culture medium delay the growth of *P. ostreatus* until approximately day 8. These results are consistent with our previous study in which we reported the growth rate of *P. ostreatus* with various concentrations of apple pomace [23]. Interestingly, the growth of *P. ostreatus* mycelia was slightly inhibited by adding 5% or 10% compared to 2.5% apple pomace. To evaluate the effect of apple pomace on laccase enzyme production, we further analyzed samples containing 0.25 µg of protein collected from the culture supernatants and supplemented with different concentrations of apple pomace (0%, 2.5%, and 5%) on different days (4, 9, and 19) using native polyacrylamide gel electrophoresis. As shown in Fig. 2, remarkable increases in laccase activities were observed in the samples with additions of 2.5% (on both days 9 and 19) and 5% (on day 19) apple pomace (Fig. 2). Even though the highest intensity was observed in the sample without apple pomace on day 4, no significant increase in activity was detected in that sample compared to the activity shown in Fig. 1.

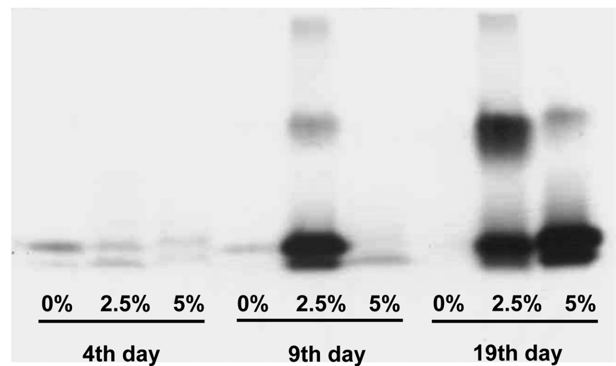

Fig. 2. Zymogram of laccase isoenzymes in culture supernatants of *Pleurotus ostreatus*. Samples containing 0.25 µg of proteins collected from the culture supernatants supplemented with different concentrations of apple pomace (0%, 2.5%, and 5%) on different days (4, 9, and 19) were used. Staining was performed with 5 mM 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) in 50 mM sodium acetate buffer (pH 5.2).

To validate and differentiate the effects of apple pomace on mRNA transcription levels of various laccase isoenzyme genes including *pox1*, *pox3*, *pox4*, *poxc* (formerly *pox2*), *poxa3*, and *poxa1b*, we conducted semi-quantitative RT-PCR. RT-PCR analyses clearly revealed that apple pomace had an effect on the induction of transcription in these laccase genes. Although the laccase activity of the sample cultured in the medium with 2.5% of apple pomace for 4 days was significantly less than those with other cultivation times (Fig. 2), RT-PCR showed the highest transcription level with 2.5% of apple pomace supplementation on day 4 (Fig. 3). Moreover, three genes (*poxc*, *poxa3*, and *poxa1b*) showed a transcription level higher than that of other genes in the cultures with 2.5% of apple pomace at all

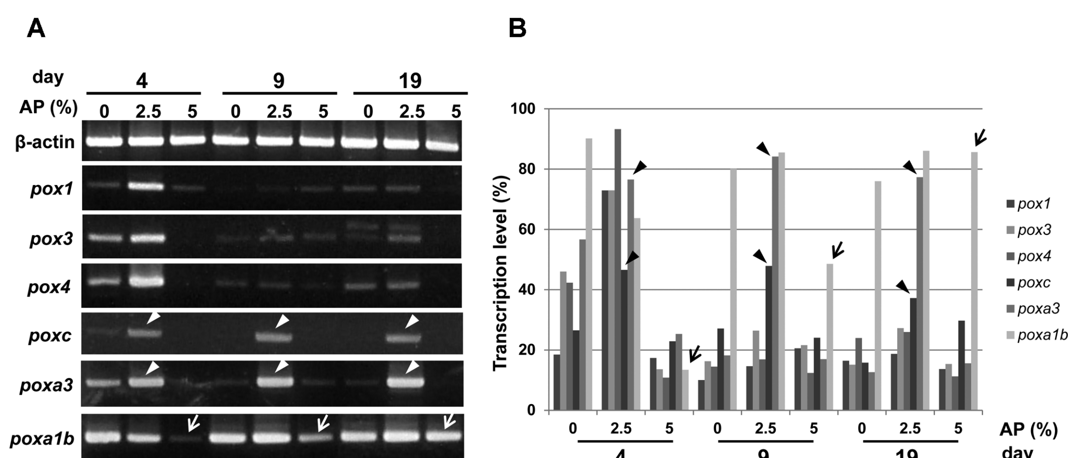

Fig. 3. Reverse transcription polymerase chain reaction assays (A) and messenger RNA transcription levels (B) of laccase isoenzyme genes of *Pleurotus ostreatus*. Total RNA was isolated from mycelia cultivated in various concentrations of apple pomace (0%, 2.5%, and 5%) at different cultivation times (days 4, 9, and 19). AP, apple pomace concentrations (w/v). Arrowheads indicate the genes (*poxc* and *poxa3*) with high transcription levels at all cultivation times with 2.5% apple pomace supplementation. Arrows indicate the gene (*poxa1b*) for which increased transcription level depends on cultivation time with 5% apple pomace supplementation.

times analyzed. In addition, the transcription level of *poxa1b* increased depending on cultivation time with 5% apple pomace supplementation. Studies of the genes coding for laccase isoenzymes in *P. ostreatus* have thus far identified four genes and their corresponding cDNAs: *poxc* (previously named *pox2*), *pox1* (coding for a laccase isoenzyme not yet identified), *poxa1b*, and *poxa3* [24]. In addition, Pezzella *et al.* [24] have isolated three new laccase genes (*pox3, pox4*, and *pox5*) from *P. ostreatus* and assessed their expression in cultures with or without the addition of inducers (copper sulfate or ferulic acid) using RT-PCR analysis. Although no RNA transcript of *pox5* could be detected, *pox3* and *pox4* were expressed under all conditions and in the presence of inducers (both copper sulfate and ferulic acid), respectively. The results, shown in Fig. 3, also revealed that *pox3* and *pox4* were expressed in cultures with and without the addition of apple pomace, although no transcript of *pox5* could be detected in culture with 5% apple pomace on both days 4 and 19. RT-PCR analyses revealed that overall high levels of transcripts were observed on day 4 of cultivation with 2.5% apple pomace. This behavior could have been due to a direct effect of apple pomace addition (with an optimal concentration of 2.5%) on laccase gene transcription during the early phase of fungal growth. However, the maximum laccase activities were observed on days 9 and 19 of cultivation with 2.5% and 5% of apple pomace, respectively, as shown in Fig. 1. High concentrations of apple pomace could result in oxidative stress (discussed above) at an advanced stage of fungal growth and could be responsible for late transcriptional induction [25]. The results of this investigation indicate that when apple pomace is added to the substrate in an attempt to increase laccase activity, its effect depends on the concentration of apple pomace.

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REFERENCES

1. Asgher M, Shah SA, Ali M, Legge RL. Decolorization of some reactive textile dyes by white rot fungi isolated in Pakistan. World J Microbiol Biotechnol 2006;22:89-93.
2. Faraco V, Pezzella C, Miele A, Giardina P, Sannia G. Bio-remediation of colored industrial wastewaters by the white-rot fungi *Phanerochaete chrysosporium* and *Pleurotus ostreatus* and their enzymes. Biodegradation 2009;20:209-20.
3. Mayer AM, Staples RC. Laccase: new functions for an old enzyme. Phytochemistry 2002;60:551-65.
4. Vyas BR, Bakowski S, Šašek V, Matucha M. Degradation of anthracene by selected white rot fungi. FEMS Microbiol Ecol 1994;14:65-70.
5. Giardina P, Aurilia V, Cannio R, Marzullo L, Amoresano A, Siciliano R, Pucci P, Sannia G. The gene, protein and glycan structures of laccase from *Pleurotus ostreatus*. Eur J Biochem 1996;235:508-15.
6. Palmieri G, Giardina P, Bianco C, Scaloni A, Capasso A, Sannia G. A novel white laccase from *Pleurotus ostreatus*. J Biol Chem 1997;272:31301-7.
7. Faraco V, Ercole C, Festa G, Giardina P, Piscitelli A, Sannia G. Heterologous expression of heterodimeric laccase from *Pleurotus ostreatus* in *Kluyveromyces lactis*. Appl Microbiol Biotechnol 2008;77:1329-35.
8. Giardina P, Autore F, Faraco V, Festa G, Palmieri G, Piscitelli A, Sannia G. Structural characterization of heterodimeric laccases from *Pleurotus ostreatus*. Appl Microbiol Biotechnol 2007;75:1293-300.

9. Palmieri G, Cennamo G, Faraco V, Amoresano A, Sannia G, Giardina P. Atypical laccase isoenzymes from copper supplemented *Pleurotus ostreatus* cultures. Enzyme Microb Technol 2003;33:220-30.
10. Giardina P, Palmieri G, Scaloni A, Fontanella B, Faraco V, Cennamo G, Sannia G. Protein and gene structure of a blue laccase from *Pleurotus ostreatus*. Biochem J 1999;341(Pt 3): 655-63.
11. Kennedy M, List D, Lu Y, Foo LY, Newman RH, Sims IM, Bain PJ, Hamilton B, Fenton G. Apple pomace and products derived from apple pomace: uses, composition and analysis. In: Linskens HF, Jackson JF, editors. Analysis of plant waste materials. Vol. 20. Berlin: Springer Berlin Heidelberg; 1999. p. 75-119.
12. Ahmad MN, Holland CR. Growth kinetics of single-cell protein in batch fermenters. J Food Eng 1995;26:443-52.
13. Dhillon GS, Brar SK, Verma M, Tyagi RD. Recent advances in citric acid bio-production and recovery. Food Bioprocess Technol 2011;4:505-29.
14. Giardina P, Cannio R, Martirani L, Marzullo L, Palmieri G, Sannia G. Cloning and sequencing of a laccase gene from the lignin-degrading basidiomycete *Pleurotus ostreatus*. Appl Environ Microbiol 1995;61:2408-13.
15. Jalč D, Nerud F, Žitňan R, Siroka P. The effect of white-rot basidiomycetes on chemical composition and *in vitro* digestibility of wheat straw. Folia Microbiol (Praha) 1996;41:73-5.
16. Villas-Bôas SG, Esposito E, Mitchell DA. Microbial conversion of lignocellulosic residues for production of animal feeds. Anim Feed Sci Technol 2002;98:1-12.
17. Tlecuitl-Beristain S, Sánchez C, Loera O, Robson GD, Díaz-Godínez G. Laccases of *Pleurotus ostreatus* observed at different phases of its growth in submerged fermentation: production of a novel laccase isoform. Mycol Res 2008;112(Pt 9):1080-4.
18. Dittmer JK, Patel NJ, Dhawale SW, Dhawale SS. Production of multiple laccase isoforms by *Phanerochaete chrysosporium* grown under nutrient sufficiency. FEMS Microbiol Lett 1997;149:65-70.
19. Moyson E, Verachtert H. Growth of higher fungi on wheat straw and their impact on the digestibility of the substrate. Appl Microbiol Biotechnol 1991;36:421-4.
20. Palmieri G, Giardina P, Bianco C, Fontanella B, Sannia G. Copper induction of laccase isoenzymes in the ligninolytic fungus *Pleurotus ostreatus*. Appl Environ Microbiol 2000; 66:920-4.
21. Collins PJ, Dobson A. Regulation of laccase gene transcription in *Trametes versicolor*. Appl Environ Microbiol 1997;63:3444-50.
22. Baldrian P. Interactions of heavy metals with white-rot fungi. Enzyme Microb Technol 2003:32:78-91.
23. Park YJ, Park HR, Kim SR, Yoon DE, Son ES, Kwon OC, Han W, Lee CS. Apple pomace increases mycelial growth of *Pleurotus ostreatus*. Afr J Microbiol Res 2012;6:1075-8.
24. Pezzella C, Autore F, Giardina P, Piscitelli A, Sannia G, Faraco V. The *Pleurotus ostreatus* laccase multi-gene family: isolation and heterologous expression of new family members. Curr Genet 2009;55:45-57.
25. Fernández-Larrea J, Stahl U. Isolation and characterization of a laccase gene from *Podospora anserina*. Mol Gen Genet 1996;252:539-51.