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Determination of lignin-modifying enzymes (LMEs) in *Hyphodermella* species using biochemical and molecular techniques

S. Rahimlou ✉
V. Babaeizad
Department of Plant Protection, Sari Agricultural sciences and Natural Resources University (SANRU), Sari, Iran

T. Bose
M. Sayari
Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa

Abstract: White-rot basidiomycetes are one of the most important lignolytic microorganisms. These fungi have been reported to secrete three main classes of lignin degrading enzymes: lignin peroxidases (LiPs), manganese peroxidases (MnPs) and laccases. In this study, for the first time the lignin degrading capability of two plant pathogens *i.e. Hyphodermella rosae* and *H. corrugata* was evaluated using both biochemical and molecular methods. Biochemical analyses included tannic acid, azur-B clearance, 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), α-naphthol and Syringaldazine test. Genes encoding for each enzyme were amplified using two pairs of degenerate primers. In the case of MnP, the primers from the previous studies failed to amplify the gene for *Hyphodermella* as well as control species, hence new primers were designed. The results from biochemical and molecular analyses confirmed the potential of both the *Hyphodermella* species to produce LiPs and MnPs, whereas they tested negative in terms of laccase production. Similar to the model white-rot fungus, *Phanerochaete chrysosporium*, laccase production may not be required for lignin decomposition by the *Hyphodermella* species treated in this study.

Key words: Laccase, Lignin peroxidase, Manganese peroxidase, Phanerochaetaceae

INTRODUCTION

After cellulose lignin is one of the most abundant and renewable carbon sources of plant origin. Biological roles of lignin include strengthening of the plant cell walls by adhesion of cellulose microfibril layers, enhancement of water transport, and protection against microbial degradation (Schmidt 2006). Due to the complexity of lignin and random phenylpropanoic polymeric structure, enzymes required for its degradation have to exhibit broad substrate specificity. Moreover, for efficient degradation, oxidases and peroxidases are preferred over hydrolases due to the high occurrence of carbon–carbon bonds (Hatakka 2001).

White-rot causing Basidiomycota are the most efficient natural degraders of lignin (Schmidt 2006). White-rot fungi are polyphyletic in origin. Hence, these fungi are distributed among all major lineages of Agaricomycetes, Basidiomycota (Hibbett et al. 2014). These fungi are characterized by their unique ability to depolymerize and mineralize lignin using extracellular enzymes. Mainly three classes of extracellular enzymes: lignin peroxidases (LiPs), manganese peroxidases (MnPs), and laccases are involved in fungal mediated lignin degradation (Schmidt 2006). Lignin peroxidase [EC 1.11.1.14] was first reported from the extracellular fluid of *Phanerochaete chrysosporium* grown under nitrogen limiting condition (Kirk & Farrell 1987). It is a monomeric haemoprotein with a molecular mass around 40 kDa (Hofrichter 2002). According to a phylogenetic study of lignolytic enzymes by Morgenstern et al. (2008), lignin peroxidases belonged to the class II secretory fungal peroxidases. Also, it was concluded that the lignin peroxidases are monophyletic in origin and diagnostic to Polyporales. The most frequent reaction catalyzed by LiP is the C_α–C_β cleavage of the propyl side-chains of lignin to give benzaldehydes using H_2O_2 as a co-substrate (Hammel & Cullen 2008). This enzyme occurs as a series of isozymes reported from several wood-decaying white-rot Basidiomycetes (Hammel & Cullen 2008).

Manganese peroxidase [EC 1.11.1.13] is another enzyme from class II secretory fungal peroxidases first reported from the extracellular fluid of lignolytic cultures of *P. chrysosporium* (Kuwahara 1984, Paszczynski 1985). MnP is glycosylated haemoprotein with a molecular mass around 45kDa (Hofrichter 2002). This enzyme is widely distributed

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✉ Corresponding authore-mail: s.rahimlou261989@gmail.com

among lignicolous fungi. Analyses by Morgenstern et al. (2008) indicated MnPs are phylogenetically older than LiPs and they are duplicated and diversified before the radiation of the major Agaricomycetes lineages. MnPs occur in at least four orders within Agaricomycetes: Polyporales, Agaricales, Corticiales and Hymenochaetales, but may also be present in Russulales (Morgenstern et al. 2008). As with the LiPs, several MnP isozymes were reported from an array of white-rot Basidiomycota (Hammel & Cullen 2008).

Laccase [EC 1.10.3.2] is one of the oldest known lignolytic enzymes, and it was first reported from Japanese lacquer tree, *Rhus venicifera* (Yoshida 1883). Laccase belongs to the large and diverse protein superfamily of multi-copper oxidases (Hatakka 2001). They catalyze numerous phenolic compounds and aromatic amines using molecular oxygen as a terminal electrons acceptor (Giardina et al. 2010). The involvement of laccase in ligninolysis is well established in *Pycnoporus cinnabarinus*, a white-rot fungus that lacks LiPs and MnPs (Eggert 1997). Several closely related laccase genes have been characterized from several lignin-degrading fungi. However, some white-rot fungi e.g. *P. chrysosporium* do not secrete laccase, suggesting that this enzyme may not be essential for lignin decay (Hatakka 1994). Also, some fungi are capable of simultaneously producing multiple laccase isoforms with different molecular weights (Hammel & Cullen 2008).

Hyphodermella is a genus of corticioid white-rot fungi from the family Phanerochaetaceae (Larsson 2007). General morphological characters of *Hyphodermella* correspond fairly well with those of *Phanerochaete* species (Wu et al. 2010). Phylogenetically *Hyphodermella* is sister clade to *Phanerochaete sensu stricto* (Floudas & Hibbett 2015, Rahimlou et al. 2015). In this study, for the first time, the lignolytic enzymes production capability of *H. rosae,* a dry fruit rot pathogen of plum and peach (Babaeizad et al. 2012, Sayari et al. 2012) as well as *H. corrugata* (ICMP 16963) was evaluated using biochemical and molecular techniques.

MATERIALS AND METHODS

Fungal specimens

In June 2011 and 2012, *Hyphodermella rosae* was isolated from symptomatic fruits of plum and peach onto Potato Dextrose Agar (PDA) medium (Merck, Germany) from Mazandaran, Iran. A combined morphological and molecular approach was used for its identification (Rahimlou et al. 2015). The isolates were deposited at the International Collection of Microorganisms from Plants (ICMP), under the accession numbers ICMP 20104 and ICMP 20105, respectively. *Hyphodermella corrugata* (Acc. No. ICMP 16963) was retrieved from ICMP.

Phanerochaete chrysosporium (PTCC 5270), *Phlebia radiata* (DSM 5111), and *Trametes versicolor* (ICMP 3069) were used as the control strains for this study. All the three control strains are known to produce LiPs and MnPs, while DSM 5111 and ICMP 3069 strains are laccase positive.

Biochemical assays

Biochemical tests specific for detection of lignolytic enzymes, *i.e.* Tannic acid, Azur-B clearance, ABTS, α-naphthol and Syringaldazine tests were applied following the protocols suggested by Pointing (1999). Tannic acid agar test (Bavendamm reaction) is not specific to any one LMEs, hence positive result provides an indication of overall polyphenol oxidase activity (Pointing 1999). It is a useful test for quick differentiation of white-rot species from the brown rots (Schmidt 2006). This test is used to confirm the potential white-rot activity of pathogenic *H. rosae* as well as *H. corrugata*. Sigma-Aldrich Corporation manufactured all the reagents used for biochemical assays.

DNA extraction and PCR amplification

Total genomic DNA was extracted from five days old culture using CTAB method (Winnepenninckx et al. 1993). Six degenerate primer pairs were used for amplification of lignin peroxidase, manganese peroxidase and laccase genes for both *H. rosae* and *H. corrugata* as well as the control species (Table 1). Primers for amplification of manganese peroxidase gene, MnPF1, MnPF2, and MnPR were designed in this study (Table 1). Individual PCR reactions were carried out using Bio-Rad MJ Mini^{TM} PTC-1148 thermal cycler. The master mix included: 10 ng of DNA, 0.1 mM dNTPs, 1 U Smart-Taq DNA polymerase (CinnaGen Co.), 4 mM $MgCl_2$, and 0.2 µM of primers in a final volume of 30 µL. The PCR conditions were adjusted to initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, respective annealing temperature for each primer pair (Table 1) for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 7 min. Positive amplifications were determined via electrophoresis which visualized using Kodak Gel Logic 200 imaging system.

RESULTS

Biochemical assays

Positive results from Tannic acid agar test indicate both *H. rosae* and *H. corrugata* belong to the white-rot group of wood decomposing fungi (Fig. 1). The disappearance of the blue color of Azur-B agar medium confirmed the peroxidase activity of the isolates (Fig. 1). Laccase production assay using ABTS, α-naphthol agar and Syringaldazine well test gave an ambiguous result. Unlike ABTS and α-naphthol, Syringaldazine tested negative for both *Hyphodermella* species (Fig. 1). Results of biochemical assays are summarized in Table 2.

Table 1. Sequences of the primers used for amplifying genes involve in lignin modification process

Primer	Sequence (5′–3′)	annealing temperature (°C)	Expected size of amplicons (bp)	Reference
Lignin peroxidase				
LgPF	GAC-GG(C/T)-CT(C/T)-GT(C/T)-CC(G/T/C)-GAG-CC	62	~ 207	Shevchenko et al. 2013
LgPR	C(A/T)G-NG(A/T/C)-CTC-GA(C/T)-GAA-GAA-CTG			
LiPF	(G/C)C(G/T/C)-AAC-AT(T/C)-GG(T/C)-CT(T/C)-GAC-GA	50	~ 450	Reddy & D'Souza 1998
LiPR	TC(G/C)-A(G/T/C)G-AAG-AAC TG(G/C)-G(A/T)G-TC			
Laccase				
LccF	GAC-AAC-(A/T)TG-ACG-AAC-CA(C/T)-ACC-ATG	52	~ 350	Shevchenko et al. 2013
LccR	CCC-CT(G/C)-A(A/G)A-CCA-TCA-CAG-TAC-TG			
LaccaseF	CA(T/C)-TGG-CA(T/C)-GGN-TT(T/C)-TT(T/C)-CA	50	~ 200	D'Souza et al. 1996
LaccaseR	(A/G)TG-(A/G)CT-(A/G)TG-(A/G)TA-CCA-(A/G)AA-(G/A/T/C)GT			
Manganese peroxidase				
MnPF1	GG(T/C)-GG(C/T/A)-GG(T/C)-GC(C/T/G)-GA(T/C)-GG(G/C)-TC	60	~ 600	In this study
MnPR	GAC-TG(G/C)-AG(G/C/A)-CG(C/G)-A(G/T)C-TCG-CC			
MnPF2	CG(T/C/G)-CT(T/C/G)-A(T/C)(T/G/C)-TTC-CAC-GAC-GC	56	~750	In this study
MnPR	GAC-TG(G/C)-AG(G/C/A)-CG(C/G)-A(G/T)C-TCG-CC			

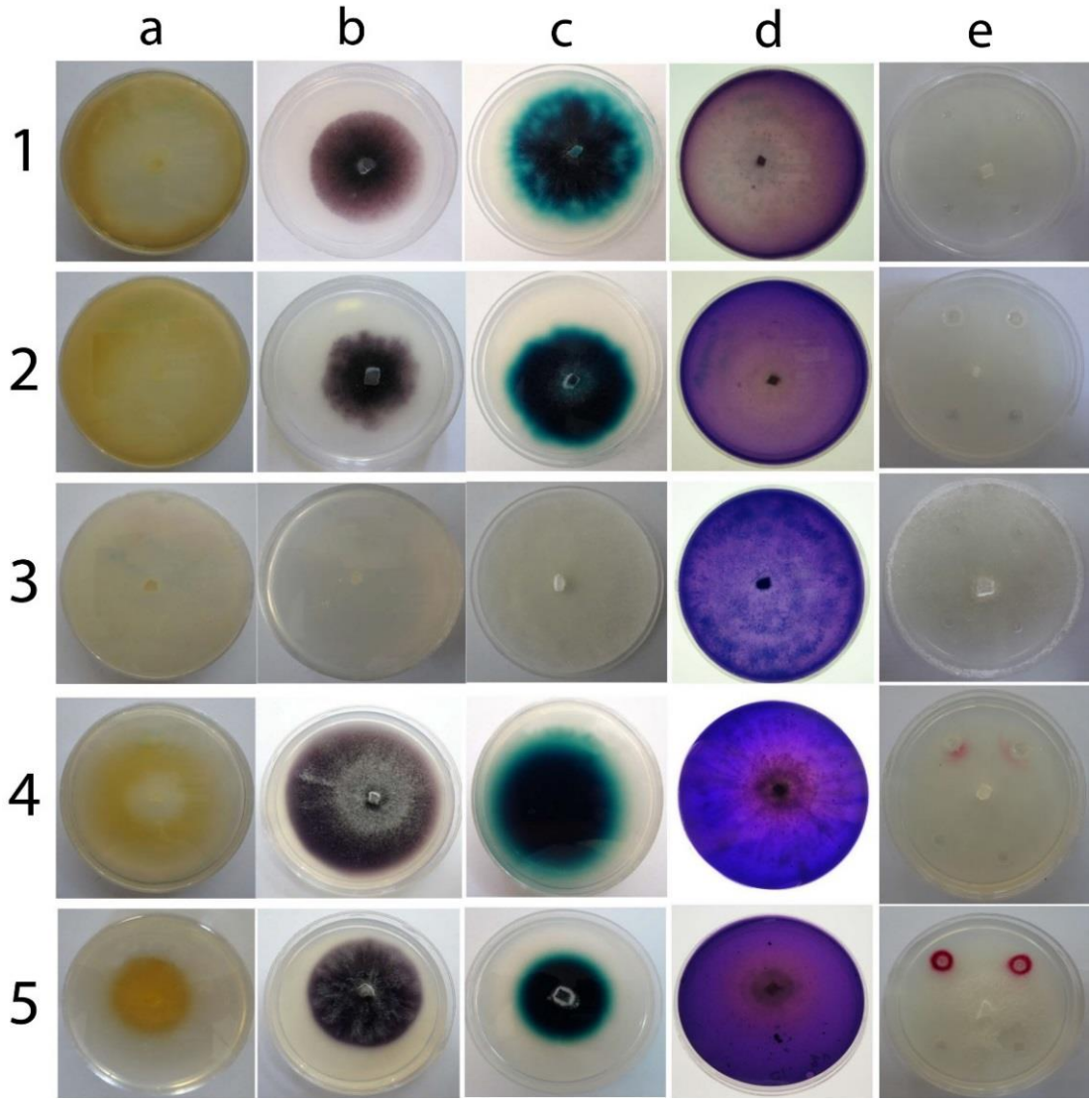

Fig 1. Biochemical assays used for LMEs determination. Tannic acid agar test, a. α-naphthol agar test, b. ABTS agar test, c. Azure-B agar clearance test, d. and Syringaldazine well test, e. *Hyphodermella rosae* (1), *H. corrugata* (2), *Phanerochaete chrysosporium* (3), *Phlebia radiata* (4) and *Trametes versicolor* (5).

PCR amplification

The genes coding for each enzyme were amplified using two pairs of degenerate primers from different loci. Amplification results were congruent with the biochemical tests for both the *Hyphodermella* species. Successful amplifications were observed for lignin peroxidase and manganese peroxidase genes (Fig. 2). Laccase gene amplification in *Hyphodermella* species failed using either of the primer pairs (Fig. 2). In some cases using a single primer pair generated two or three amplicons with an approximately 50-100 bp difference in size, which might be due to the several duplications of the gene.

Table 2. Results of biochemical assays used to indicate lignolytic enzymes production

Biochemical tests	Tannic acid	Azur-B clearance	ABTS	α-naphthol	Syringaldazine
Hyphodermella rosae	P	P	P	P	N
Hyphodermella corrugata	P	P	P	P	N
Phlebia radiata	P	P	P	P	P
Phanerochaete chrysosporium	N	P	N	N	N
Trametes versicolor	P	P	P	P	P

P: Positive, N: Negative

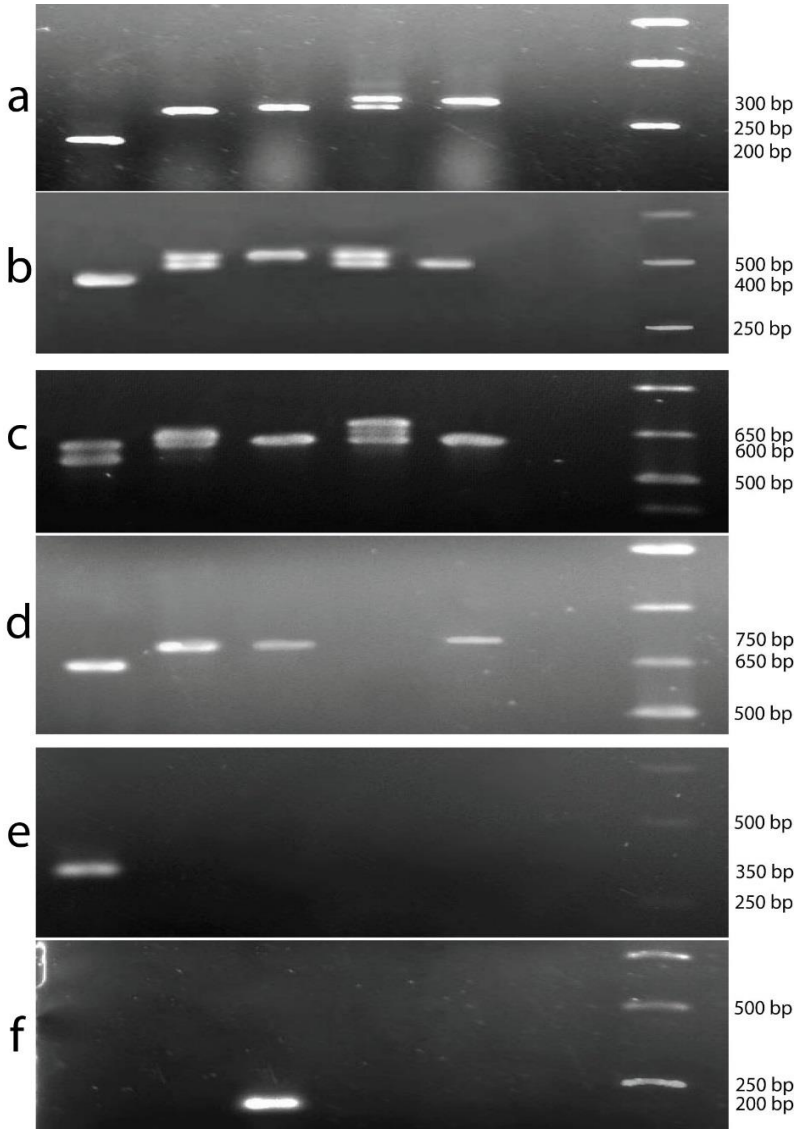

Fig. 2. Gel electrophoresis of PCR products. LgP gene amplification using LgPF/LgPR (a) and LiPF/LiPR (b) primer pairs. a. Left to right: *Trametes versicolor*, *Phanerochaete chrysosporium*, *Phlebia radiata*, *Hyphodermella corrugata*, *H. rosae*, control reaction mixture without DNA, DNA ladder. b. Left to right: *T. versicolor*, *P. radiata*, *Phanerochaete chrysosporium*, *H. corrugata*, *H. rosae*, control reaction mixture without DNA, DNA ladder. MnP gene amplification using MnPF1/MnPR (c) and MnPF2/MnPR (d) primer pairs. c. Left to right: *T. versicolor*, *P. chrysosporium*, *Phlebia radiata*, *H. corrugata*, *H. rosae*, control reaction mixture without DNA, DNA ladder. d. Left to right: *T. versicolor*, *Phanerochaete chrysosporium*, *Phlebia radiata*, *H. corrugata*, *H. rosae*, control reaction mixture without DNA, DNA ladder. Laccase gene amplification using LccF/LccR (e) and LaccaseF/LaccaseR (f) primer pairs. e, f. Only in case of *T. versicolor* amplicons generated by using both primer pairs.

DISCUSSION

Laccase is one of the most widely distributed lignolytic enzymes and has been reported from all major clades of the kingdom fungi. Hence, we emphasized to see if *Hyphodermella* like many other white-rot fungi has the ability to produce laccase. Detection of laccase using biochemical tests is often confusing. ABTS and α-naphthol assays are useful for detecting laccase activity, although the results should be interpreted cautiously as peroxidase enzymes are also known to oxidize ABTS and α-naphthol in the presence of H_2O_2. Positive results from these tests should only be accepted if a negative reaction is obtained using a peroxidase specific growth medium such as Azur-B agar (Pointing 1999).

Both the *Hyphodermella* species tested for positive peroxidase activity using ABTS, α-naphthol and Azur-B agar. Hence, Syringaldazine well test a specific substrate for determining laccase activity was used. Negative result from Syringaldazine test as well as PCR amplification confirmed the absence of laccase activity for both the *Hyphodermella* species. Based on these observations we hypothesize that similar to *Phanerochaete*, *Hyphodermella* also deficits laccase activity as they represent the same family Phanerochaetaceae. Alternatively, the primers were not suitable for amplifying the laccase gene for *Hyphodermella*. The first hypothesis seems to have stronger probability, because both *Hyphodermella* and *Phanerochaete* also tested negative for Syringaldazine reaction.

In case of enzyme manganese peroxidase, primers designed by Shevchenko et al. (2013) did not give satisfactory amplification. This observation is in line with the study by Pointing et al. (2005). In this study, Pointing and his colleagues failed to amplify the MnP gene from basidiomycetous fungi using the primers suggested by Bogan et al. (1996). They concluded that the designed primers were inappropriate for amplifying MnP genes from a wide range of taxa, because they were designed based on a single MnP isozyme from *P. chrysosporium*. This led us to design MnPF1, MnPF2 and MnPR primers based on several isozyme sequences of MnP from *T. versicolor*, *P. chrysosporium*, *P. radiata* and *P. sordida.* Primer pair MnPF1/MnPR successfully amplified the *MnP* genes for both the *Hyphodermella* species as well as the controls while MnPF2/MnPR failed to amplify *H. corrugata.*

Evidences from this study unveils the ability of *Hyphodermella* spp. to produce ligninolytic enzymes, LiPs and MnPs, which were unknown until now. Even though degenerate primers were used still we couldn't sequence the amplicon. This might be due to presence of several homologous genes for each enzyme, which is exemplified by several amplicons of similar sizes generated during a single PCR reaction. Hence further investigation is required in order to detect isoforms for each enzymes and to sequence the corresponding genes.

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تعیین تولید آنزیم های تجزیه کننده لیگنین در گونه های قارچ *Hyphodermella* با استفاده از روش های بیوشیمیایی و ملکولی

صالح رحیم لو[1] ✉، ولی اله بابایی زاد[1]، تانای بس[2] و محمد سیاری[2]

۱– گروه گیاهپزشکی، دانشگاه علوم کشاورزی و منابع طبیعی ساری، ایران

۲– موسسه بیوتکنولوژی کشاورزی و جنگلداری، دانشگاه پرتوریا، آفریقای جنوبی

چکیده: بازیدیومیست های عامل پوسیدگی سفید یکی از مهمترین میکروارگانیسم های تجزیه کننده لیگنین هستند. گزارش شده است که این قارچ ها سه گروه مهم از آنزیم های تجزیه کننده لیگنین که عبارتند از: لیگنین پراکسیداز (LiPs)، منگنز پراکسیداز (MnPs) و لاکاز را تولید می کنند. در این مطالعه، برای اولین بار توانایی تجزیه لیگنین در دو بیمارگر گیاهی *Hyphodermella rosae* و *H. corrugata* با استفاده از روش های بیوشیمیایی و ملکولی ارزیابی شد. آنالیز بیوشیمیایی شامل آزمون های Tannic acid، Azur–B clearance ، (ABTS) 2,2'–azino–bis(3–ethylbenzothiazoline–6–sulphonic acid)، α– naphthol و Syringaldazine بودند. ژن های کد کننده هر آنزیم با استفاده از دو جفت آغازگر دژنره تکثیر شدند. در مورد آنزیم منگنز پراکسیداز، آغازگرهای استفاده شده در مطالعات قبلی موفق به تکثیر این ژن در گونه های *Hyphodermella* و همچنین در گونه های شاهد نشدند. بدین منظور، آغازگرهای جدید در این مطالعه طراحی شد. نتایج آنالیزهای بیوشیمیایی و ملکولی نشان داد که گونه های *Hyphodermella* دارای پتانسیل تولید آنزیم های لیگنین پراکسیداز و منگنز پراکسیداز هستند، در حالیکه، نتیجه برای تولید لاکاز منفی بود. به نظر می رسد که تولید لاکاز برای تجزیه لیگنین در گونه های *Hyphodermella* همانند گونه *Phanerochaete chrysosporium* که یک قارچ مدل در ایجاد پوسیدگی سفید است، مورد نیاز نمی باشد.

کلمات کلیدی: لاکاز، لیگنین پراکسیداز، منگنز پراکسیداز، Phanerochaetaceae

مکاتبه کننده: صالح رحیم لو Email: s.rahimlou261989@gmail.com

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