DOI: https://doi.org/10.36036/MR.34.1.2025.168871
Mushroom Research 34 (1) : 97-106, 2025

Expression profiling of laccase and peroxidase genes in *Schizophyllum commune* during different development stages

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ABSTRACT

***Schizophyllum commune* is a white-rot fungus that can efficiently degrade lignin into low-molecular-weight compounds by producing various extracellular lignolytic enzymes, such as laccase and peroxidase. In a previous study, *S. commune* strain DMRX-2160 was identified as the best *S. commune* strain in terms of maximum extracellular enzyme activities during both submerged cultivation and solid-state fermentation. The changes in lignolytic activities across different growth stages, from mycelial run to sporophore formation, was analyzed through gene expression studies of DMRX-2160 cultivated in paddy straw using real-time PCR. Laccase and peroxidase genes were found maximally expressed in the mycelium of DMRX-2160 (42.52 fold for laccase and 4.86 fold for peroxidase than young fruiting body stage). The expression of these genes showed downregulation over the subsequent developmental stages with its lowest expression in young fruiting body stage. The maximum expression of the lignolytic enzyme during the mycelial stage decreased during the fruiting body stage, indicating its increased ability to utilize more nutrients from the substrate degradation.**

Keywords: *Schizophyllum commune*, laccase, peroxidase, Real time PCR

The white rot mushrooms can break down all parts of plants, including cellulose, hemicellulose and lignin (Martinez *et al.,* 2005). They can degrade lignin efficiently into low molecular weight compounds since they produce various extracellular lignolytic enzymes *viz.*, laccase (EC 1.10.3.2), lignin peroxidase (EC 1.11.1.14) and manganese peroxidase (EC 1.11.1.13) (Ardon *et al.,* 1996). Laccase enzyme oxidizes phenols and catalyzes the one-electron oxidation of ortho/ para diphenols and aromatic amines, thus it breaks down lignin (Patel and Gupte, 2016). Peroxidases are extracellular glycosylated heme proteins, which catalyse the H_2O_2-dependent one-electron oxidation of a range of aromatic compounds related to lignin. These lignolytic enzymes have potential applications in different industries like food, textile, dyes, bioremediation, cosmetics, analytical biochemistry, paper and pulp (Arabi *et al.,* 2011).

Schizophyllum commune also known as split gill mushroom belongs to the category of white rot fungi and is a naturally occurring medicinal mushroom that grows on rotting wood. *S. commune* is considered as an excellent source of laccase and peroxidase enzyme, as it is a lignin-degrading fungus with an active lignocellulolytic machinery (Thiribhuvanamala *et al.,* 2020). Among three strains of this mushroom experimented, DMRX-2160 was identified as the best *S. commune* strain, in terms of maximum extracellular enzyme activities during submerged cultivation and solid-state cultivation (Arya *et al.,* 2024). The efficient production of mushroom depends on precise control

Recieved on 10-07-2025; Accepted on 24-09-2025
https://epubs.icar.org.in/index.php/MR/article/view/168871

of fruiting body development through lignin degradation and identification of the molecular mechanism behind it has commercial and scientific significance (Nesma *et al.,* 2023). The genes involved in the lignin breakdown and fruiting body formation can be elucidated by transcriptome and comparative expression analysis at various developmental stages (Fu *et al.,* 2017).

The present study was undertaken for expression profiling of laccase and peroxidase genes of *S. commune* strain at different developmental stages by qualitative real-time PCR, to evaluate its lignin degradation potential at the genetic level. The changes in lignolytic activities over the growth stages *viz.*, mycelia, primordia and young fruiting body of *S. commune* strain DMRX-2160 cultivated in paddy straw was analyzed by gene expression studies.

MATERIALS AND METHODS

Cultivation of *Schizophyllum commune* strain DMRX-2160 using solid substrates

Paddy grains were used as the substrate for spawn production. The grains were thoroughly washed three to four times in clean water, followed by overnight soaking in water. After draining off surplus water, the grains were then spread on a clean surface for drying. The surface dried grains were combined with calcium carbonate at the rate of 50 g/kg and were packed in polypropylene bags (12x6 inch) at the rate of 300 g per bag, followed by autoclaving at 121^{0}C and 1.02 kg/cm^2 pressure for 2 h. After cooling, each bag was aseptically inoculated with mycelial bits from a 10-day-old culture plate of *S. commune* strains and incubated at 28±2°C, until the mycelium completely colonized the grains.

Mushroom beds were prepared using paddy straw as substrate. Fresh paddy straw was soaked in water containing 75 ppm carbendazim (bavistin) and 500 ppm formalin for 18 h for chemical sterilization. The straw was sun-dried until the moisture level dropped to sixty percent. The mushroom beds were prepared using polypropylene bags of 60 x 30 cm size (thickness of 100 mm gauge), and pressed with hand for making it even. A small amount of spawn about 15 g was sprinkled and another layer of paddy straw was placed over the spawn and gently compacted. Repeating this process three or four times, the mouth of the bag was sealed and rolled. Aeration was achieved by making holes in the bag. Inoculated paddy straw bags were incubated in a dark ventilated room at 25°C for 15-20 days. Following the spawn run, slits were put in polybags to allow pinheads emergence. After that, the beds were subsequently transferred to the cropping room, where water was sprayed to maintain the temperature and relative humidity at 28±2°C and 85 to 90 per cent, respectively.

Sample collection

Samples from three distinct developmental stages *viz.*, mycelia (M), primordia (P) and young fruiting body (Yfb) of *S. commune* strain DMRX-2160 were used for the study.

RNA Isolation

The total RNA was isolated from the samples at different developmental stages of *S. commune* strain DMRX-2160 by a modified protocol using TRIzol reagent (Chomczynski *et al.,* 1995). Agarose gel electrophoresis was performed to determine the quality and integrity of isolated RNA. The purity as well as concentration of isolated RNA was determined spectrophotometrically using the ratio of absorbance at 260 nm and 280 nm (A_{260}/A_{280}). The concentration of RNA in the sample was calculated using the formula:

Concentration of RNA (ng/μl) = A_{260} x 40 x dilution factor.

cDNA synthesis

cDNA synthesis was done according to the manufacturer's instructions (AURA BIOTECH, Chennai India). The composition of 20 µl reaction mixture made for cDNA synthesis is given in Table 1 and 2. The preincubation reaction mixture was heated at 65°C followed by incubation for 5 min and the tubes were placed immediately on ice. To this the rest of components for cDNA synthesis were added. After mixing the contents well, it was incubated at 42°C for 60 min. To inactivate the RT enzyme, it was kept for another incubation at 85°C for 5 min (Table 3) and finally, the cDNA samples obtained were stored at -20°C for further use. Confirmation of cDNA synthesized was done by PCR using reference gene β-actin primer.

Table 1. Reaction mix used for pre-incubation of cDNA synthesis

Reagents	Volume
Oligo(dT) (50 µM)	2 µl
Template (RNA) (1 µg)	X µl
dNTPs (10 mM)	1 µl
RNase-Free ddH_2O	Up to 10 µl
Total volume	10 µl

Table 2. Reaction mix used for cDNA synthesis

Reagent	Volume
5x RT reaction buffer	4.0 µl
Reverse transciptase (200 U/µl)	1 µl
RNase Inhibitor (40 U/µl)	0.2 µl
dNTPs (10 mM)	1 µl
RNase-Free ddH_2O	3.8 µl
Total volume	10 µl

Table 3. Thermal cycler condition used for cDNA synthesis

Steps	Temperature (^{0}C)	Time (min)
Reverse transcription	42^{0}C	60
Inactivation	85^{0}C	5

Designing of Primer

The nucleotide sequences of genes such as laccase and heme peroxidase of *S. commune* were available in the NCBI database. The sequences retrieved from GenBank were used for designing gene-specific primers. The primer designing was done using "Primer 3 Plus" software. The β-Actin gene was used as the reference gene for Real-Time PCR studies (Madhavan *et al.,* 2014).

Standardization of annealing temperature

A gradient PCR was carried out for all primers at different annealing temperatures ranging from 56°C to 60°C to standardize the optimum annealing temperature (Ta). A reaction mixture of 25 µl was prepared as per Table 4. PCR amplification was performed using Rotor-Gene Q 5plex (QIAGEN, USA). The thermal profile for Ta standardization is given in Table 5.

Table 4. Reaction mix used for Ta standardization

Reagent	Volume
2X Premix (TB Green a! from Takara)	12.5 µl
Forward primer	0.5 µl
Reverse primer	0.5 µl
cDNA (1:5)	2 µl
ddH_2O	9.5 µl
Total volume	25 µl

Table 5. Thermal profile for Ta standardization

Steps	Cycle	Temperature (°C)	Time (s)
Initial denaturation	1	95°C	300
Denaturation		95°C	30
Annealing	35	56°C, 57°C, 59°C and 60°C	30
Extension		72°C	45

Real-Time PCR analysis

qRT-PCR analysis was performed using β-actin gene as the endogenous control to study the differential expression of laccase and peroxidase genes in *S. commune* strain DMRX-2160 during different developmental stages. The cDNA prepared from samples at each developmental stage of the mushroom was used for the exponential amplification, performed in the Rotor-Gene Q 5plex (QIAGEN, USA) Real-Time PCR system. An intercalating dye called SYBR green, was used for generating fluorescence. The composition of reaction mixture (25 µl) and thermal profile for qRT-PCR are given in Table 6 and Table 7, respectively. For each gene, qRT-PCR was performed in triplicates with a reaction volume of 25 µl for each sample. A non-template control (NTC) was also used in all the reactions as negative control. The Ct values obtained from qRT-PCR were used for further analysis. Analysis of the relative fold change in gene expression was done using the Comparative $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Table 6. Reaction mix used for qPCR analysis

Reagent	Concentration	Volume
2X TB Green a! SYBR Green qPCR Mix	2X	12.5 µl
Forward primer	10 pmol	0.5 µl
Reverse primer	10 pmol	0.5 µl
cDNA (1:5)	—	2 µl
DNase free water	—	9.5 µl
Total volume		25 µl

Table 7. Thermal profile used for qPCR analysis

Steps	Cycle	Temperature (°C)	Time (s)
Initial denaturation	1	95°C	300
Denaturation		95°C	30
Annealing	35	60°C (Laccase) 56°C (Peroxidase) 60°C (β-actin)	30
Extension		72°C	45

RESULTS AND DISCUSSION

Mycelial growth of *Schizophyllum commune* strain DMRX-2160 was observed three days. The spawn run in the substrate took 6-7 days for completion, pinheads initiation took 9-12 days and mature fruit bodies could be harvested after 15 days (Fig. 1). The samples were collected at mycelia (M), primordia (P) and young fruiting body stages for RNA extraction and Quantitative real time PCR analysis.

Isolated RNA from three different developmental stages of DMRX-2160 *viz.*, mycelium, primordium and young fruiting body is depicted in Fig. 3 and concentration of RNA is shown in Table 8.

The synthesized cDNA was confirmed by PCR using reference gene primer. The gene sequence of peroxidase (heme peroxidase) of *S. commune* was retrieved from GenBank for designing gene-specific primers, however, predesigned primer for laccase gene of *S. commune* was obtained from literature search. The ideal primer length is between 18 and 24 bp with a suitable GC content of 45-60%. In the present study, good quality gene-specific primers for peroxidase gene was designed using "Primer 3 plus" software. β-Actin gene primer was chosen from the article by Madhavan *et al.* (2014). The details of the primers are given in Table 9.

The optimum annealing temperature was found to be 60°C for laccase and â-actin genes; and 56°C for peroxidase gene on performing gradient PCR from 56°C to 60°C. PCR was performed using the synthesized cDNA with gene-specific primers to

Fig. 1. Developmental morphology of DMRX-2160 in beds prepared with paddy straw

Fig. 2. Samples collected from developmental stages of *S. commune* strain DMRX-2160 for RNA extraction

check the specificity of the primers designed for laccase and peroxidase genes. The differential expression of laccase and peroxidase genes of DMRX-2160 during different developmental stages was carried out by quantitative Real-Time PCR (qRT-PCR).

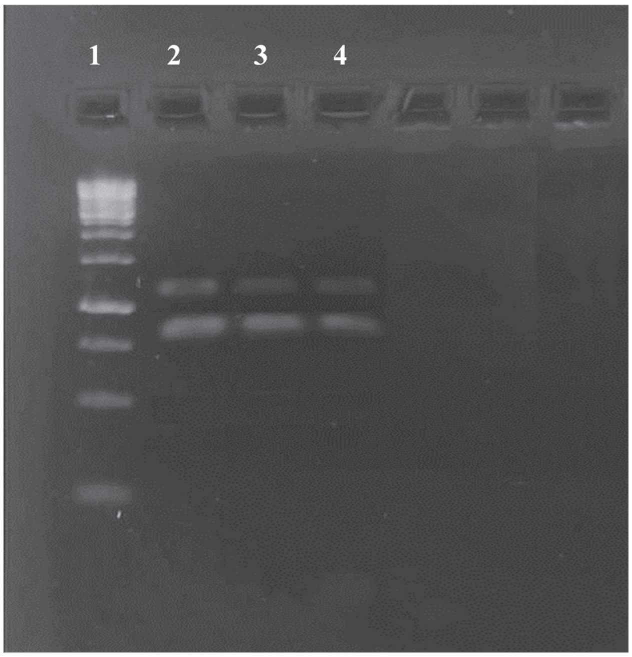

Fig. 3. Gel profile of RNA isolated from DMRX-2160 (Lane 1: 1 kb marker; Lane 2-4: RNA from mycelia, primordia, young fruiting body of DMRX-2160)

Table 8. Concentration and purity of RNA

Samples	Concentration (ng/µl)	A260/A280
Mycelium	192.23	1.88
Primordium	171.03	1.95
Young fruiting body	168.20	1.98

Table 9. List of primers used for Real-Time PCR studies

Gene	Primer sequence (5'-3')	Length (bp)
Laccase *(Lacc1)*	F 5´ GACAGCACGCTATTCAATG GCAAG 3´	24
	R 5´ CTCGTTGACCATCAAAGGT TCCGT 3´	24
Peroxidase	F 5' CATCCGAGGAGATGATTGGT 3'	20
	R 5' TGCCCTCGATGTACTCTGTG 3'	20
β-actin	F 5´ CTGCTCTTGTTATTGACAATG GTTCC 3´	26
	R 5´ AGGATACCACGCTTGGACTG AGC 3´	23

qRT-PCR for each gene was performed in triplicates for each sample. Amplification plots were generated by the Rotor-Gene Q 5plex (QIAGEN, USA) Real-Time PCR system (Fig. 4). The Ct values generated by qRT-PCR were used for further analysis. Average Ct values obtained are given in Table 10. The relative fold change in gene expression with respect to the young fruiting body stage (taken as control) for each strain are detailed in Table 11. The normalized expression values in the present study indicated that the expression pattern of laccase (*Lacc1*) of DMRX-2160 showed variation during different developmental stages. Highest expression of the gene was noticed in the mycelial stage which was 42.52-fold more than the young fruiting body stage and got downregulated over the subsequent developmental stages (Fig. 5). Similarly, the expression pattern of peroxidase gene also showed variation during different developmental stages in DMRX-2160. Peroxidase gene was 4.86-fold upregulated in mycelium than the young fruiting body stage and got downregulated over the subsequent developmental stages.

Table 10. Average Ct values of genes in S. commune strain DMRX-2160 at different developmental stages

Sample	Ct average		
	Laccase	Peroxidase	β-actin
Mycelia	26.17	24.53	21.24
Primordia	29.02	25.12	21.13
Young fruiting body	31.09	26.31	20.75

Table 11. Relative fold change in gene expression of *S. commune* strain DMRX-2160 at different developmental stages

Sample	Relative fold change	
	Laccase	Peroxidase
Mycelia	42.52	4.86
Primordia	5.48	2.98
Young fruiting body	1.00	1.00

The transition from mycelium to primordium is a crucial and challenging developmental process for fruiting body formation. The maximum expression of the lignolytic enzyme during mycelial growth which decreased over fruiting body stage of the mushroom indicated its ability to utilize more nutrients from the substrate (paddy straw) through degradation and thus facilitate primordial initiation (Nesma *et al.,* 2023). This was the reason for the higher yield of DMRX-2160, with highest lignolytic potential, when compared to other two strains of *Schizophyllum commune*.

A similar expression profile of laccase transcription has been reported in *Agaricus bisporus* and *Lentinula edodes* cultivated in wheat straw, by Pezzella *et al.* (2013), in which the level of laccase transcripts was highest in the mycelial growth phase, which rapidly decreased at the beginning of fructification. Similar result was obtained for Madhavan *et al.* (2014) who analysed the differential regulation of laccase and laccase-like MCOs genes in *S. commune grown* in solid minimal media*,* at various morphogenetic stages of sexual development. They reported that, laccase genes *viz.*, *lcc2* and *mco1* showed four-fold and two-fold upregulation, respectively, in the dikaryotic phase than fruiting body stage, however, the expression of gene *mco2* was up-regulated in the primordial phase with a slight reduction at the fruiting phase. Castanera *et al.* (2015) found peak expression level of gene *lacc10* during the initial stages of growth (day 3) corresponding to the mycelia of *Pleurotus ostreatus* on semi solid substrate fermentation (s-SSF) medium of wheat straw and wood chips. It was followed by a gradual decrease in expression over the developmental stages with the least expression in the final stage of growth (day 30), which is the fruiting body stage. They also explained that higher level of lignolytic enzyme production in lignocellulose-based SSF media when compared to glucose-based SmF (submerged fermentation) medium was due to the lignin-rich environment. Hua *et al.* (2018) analysed the expression profile of *lacc10* in *Pleurotus tuoliensis* and reported that the transcript level was downregulated at beginning of fructification stage and significantly decreased during fructification.

Highest expressions (more than 10 fold upregulation) of laccase and laccase like multicopper oxidase genes, were reported in dikaryotic phase of *S. commune* cultivated on black slate and wood by Kirtzel *et al.* (2018) and Krause *et al*. (2020). The results of expression analysis of the present study are in accordance with the findings of Nesma *et al.* (2023) who also reported higher expression of laccase gene *(POXA3)* in the mycelial stage (DMRP115-5.58 fold and HUC-2.03 fold) over fruit body stage in *Pleurotus ostreatus* DMRP-115 and HUC strains cultivated on rubber wood sawdust.

S. commune is a white rot fungus which degrades all woody cell wall com-ponents including lignin. Lignin-degrading enzymes, which are commonly classified as FOLymes, com-prise of lignin oxidases (LO families) which in turn consists of laccases (LO1), lignin peroxidases, manganese peroxidases, versatile peroxidases (LO2) and cellobiose dehydrogenases (CDHs; LO3). *S. commune* contains 16 FOLyme genes, however, its genome lacks genes encoding peroxidases of the LO2 family (Ohm *et al*., 2010). So, our study is the first report on the expression analysis of peroxidase gene at different developmental stages of *Schizophyllum commune* strain. The maximum expression of the lignolytic enzyme during mycelial stage decreased over fruiting body stage indicated by its ability to utilize more nutrients from the substrate through degradation. This facilitated primordial initiation and fruiting body development, which can be attributed for the higher yield of DMRX-2160 with highest lignolytic potential. The obtained gene expression pattern correlated with the levels of extracellular and intracellular enzyme activities expressed by *Schizophyllum* strain. Findings from the present study pointed out that *S. commune*

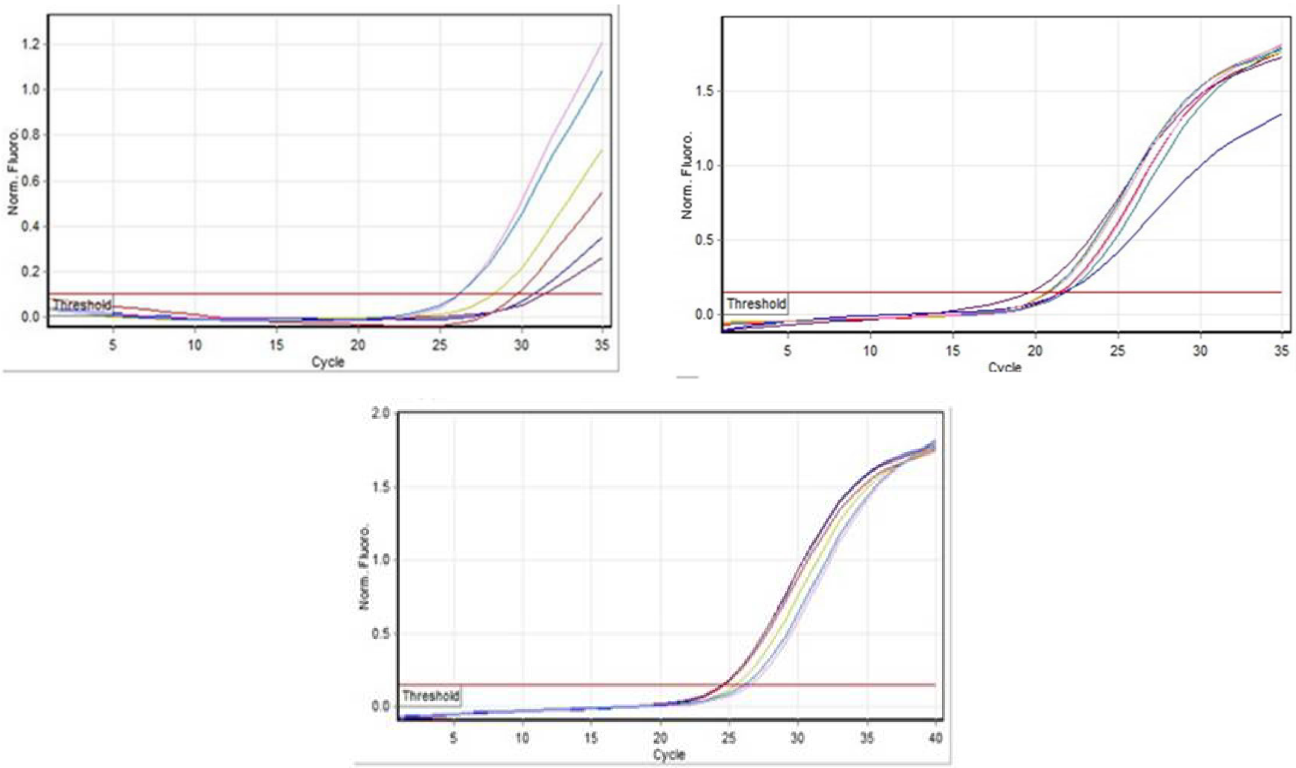

Fig. 4. Amplification curve analysis: (a) β-actin; (b) Laccase; (c) Peroxidase

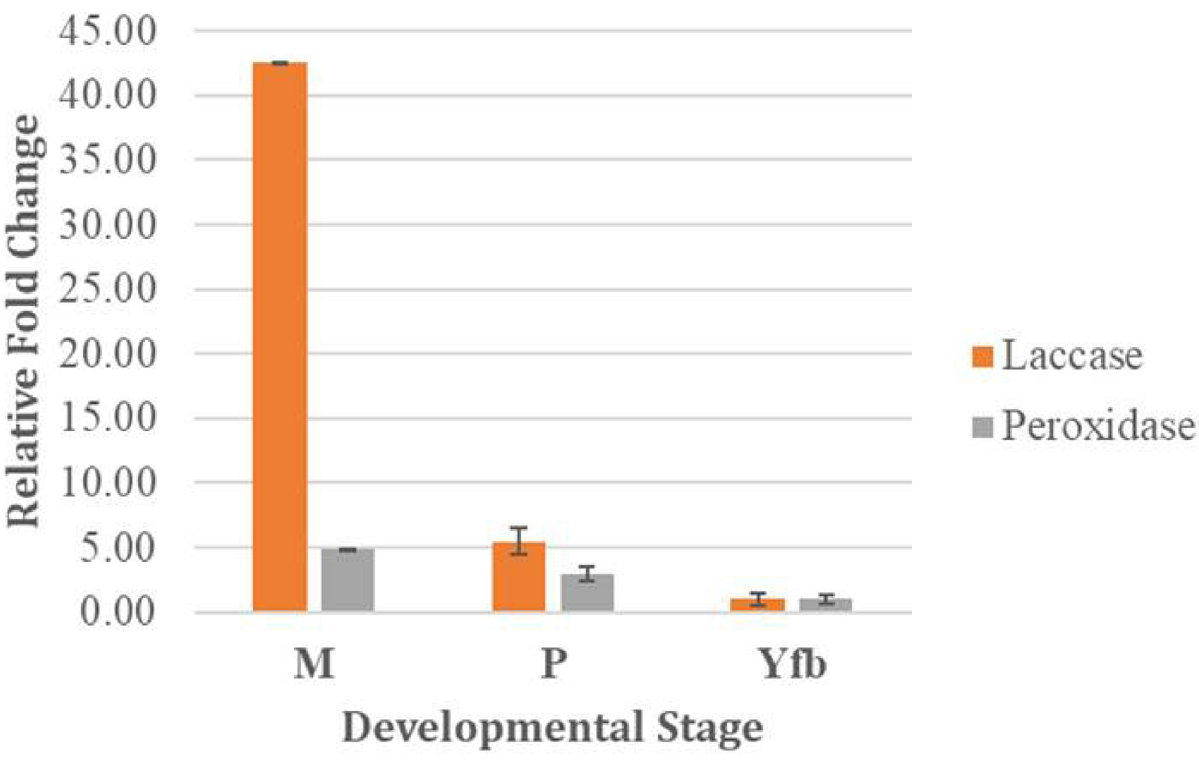

Fig. 5. Differential expression of laccase and peroxidase genes at different developmental stages of DMRX-2160 (M – Mycelia, P – Primordia, Yfb – Young fruiting body)

strain DMRX-2160 is a prospective enzyme source for the extraction of lignolytic enzymes *viz*., laccase and peroxidase having biotechnological and industrial applications.

ACKNOWLEDGEMENTS

The authors express their sincere gratitude to Kerala Agricultural University for their financial support, as well as to the Department of Plant Pathology at the College of Agriculture, Vellayani, for providing essential facilities and technical assistance. The authors declare no conflict of interest related to this report or the study.

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