Effect of Different Inducers on Laccase Production in Perenniporia sp. Isolated from Bukit Tangkiling Nature Park, Central Kalimantan

Abudarin
Study Program of Chemistry Education, Postgraduate Program, Universitas Palangka Raya

I Nyoman Sudyana
Study Program of Chemistry Education, Postgraduate Program, Universitas Palangka Raya

Soaloon Sinaga
Study Program of Biology Education, Postgraduate Program, Universitas Palangka Raya

Wahyu Supriyati
Study Program of Biology Education, Postgraduate Program, Universitas Palangka Raya

他

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Effect of Different Inducers on Laccase Production in *Perenniporia* sp. Isolated from Bukit Tangkiling Nature Park, Central Kalimantan

Abudarin[1], I Nyoman Sudyana[1,2], Soaloon Sinaga[3], Wahyu Supriyati[3], Desi Panjaitan[4], Karelius[2], Dessy Analinta[1], Sugeng Mashabhi[3], Cindi Siagian[4], Retno Agnestisia[1,2,*]

[1]Study Program of Chemistry Education, Postgraduate Program, Universitas Palangka Raya, Jl. Yos Sudarso, Palangka, Kec. Jekan Raya, Kota Palangka Raya, Kalimantan Tengah 73112, Indonesia

[2]Study Program of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Palangka Raya, Jl. Yos Sudarso, Palangka, Kec. Jekan Raya, Kota Palangka Raya, Kalimantan Tengah 73112, Indonesia

[3]Study Program of Biology Education, Postgraduate Program, Universitas Palangka Raya, Jl. Yos Sudarso, Palangka, Kec. Jekan Raya, Kota Palangka Raya, Kalimantan Tengah 73112, Indonesia

[4]Study Program of Biology, Faculty of Mathematics and Natural Sciences, Universitas Palangka Raya, Jl. Yos Sudarso, Palangka, Kec. Jekan Raya, Kota Palangka Raya, Kalimantan Tengah 73112, Indonesia

*Author to whom correspondence should be addressed:
E-mail: retno.agnestisia@mipa.upr.ac.id

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Abstract: Laccase (p-diphenol: dioxygen oxidoreductase) is a lignin-degrading enzyme widely found in white rot fungi and has been utilized in various industrial and environmental applications. Here, P*erenniporia* sp., a white rot fungus from the Bukit Tangkiling Nature Park, Central Kalimantan (sample code CS23062), was collected and isolated from its fruiting body using the surface sterilization method on potato dextrose agar (PDA) medium. The fungal isolate was molecularly recognized via internal transcribed spacer (ITS) sequences of the ribosomal DNA region through the polymerase chain reaction (PCR) technique. Then, the laccase performance of the fungal isolate was investigated via PDA supplemented with 0.02% guaiacol. Subsequently, the isolate was cultured in a nitrogen-limited medium with different inducers to enhance laccase production. Laccase activity was investigated spectrophotometrically by observing the oxidation of the syringaldazine substrate. The result revealed that a nitrogen-limited medium added with veratryl alcohol inducer could improve laccase production in the P*erenniporia* sp. isolate.

Keywords: Laccase; *Perenniporia* sp.; white rot fungus; inducer; production

1. Introduction

Laccase is a multicopper oxidase found extensively in higher plants, insects, bacteria, and fungi[1–3]. Laccases generally play a role in various biometabolic processes, including those related to fungal pigmentation and lignin biodegradation[1]. In lignin biodegradation, the phenolic lignin oxidation was facilitated by this enzyme utilizing molecular oxygen (O_2) as an electron acceptor[8,9]. The use of O_2 as an electron acceptor is considered more cost-effective due to its abundance in nature. Laccase can also oxidize non-phenolic lignin by utilizing appropriate redox mediators[10–12]. Besides, laccase is also capable of oxidizing various types of substrates[1].

According to the benefits, laccase has recently become a concern of extensive studies, especially fungal laccases. They have relatively high redox potentials, are secreted extracellularly, and can be easily purified, offering significant advantages for industrial and environmental applications[1]. Fungal laccases are frequently obtained from Ascomycota and Basidiomycota fungi[1,4,5]. One example is *Perenniporia* sp., a white rot fungus from the basidiomycete group, which is capable of producing laccase enzymes[6,7]. This fungus is also one of the dominant basidiomycete fungi in Tangkiling Hill Natural Park, Central Kalimantan, Indonesia, a region that has not been extensively explored for its fungal diversity. The unique ecological conditions of this habitat, including high humidity and abundant decaying wood, create an ideal environment for ligninolytic fungi, which play a crucial role in organic matter decomposition.

In particular, the nitrogen-limited medium is a culture

medium generally employed to produce laccase from numerous white rot fungi[13], such as *Trametes* sp.[14], *Trametes versicolor*[15,16], *Coriolopsis polyzona*[15], *Pleurotus ostreatus*[17], and *Phlebia floridensis*[18]. In addition, many researchers have reported that organic compounds given to the culture medium function as inducers stimulating laccase production[19–24]. Lee et al. (1999) exposed that the production of 20-fold laccase from *T. versicolor* could be improved by adding ethanol inducer[19]. Besides, it was found that verartyl alcohol could most effectively induce the laccase production from *T. vesicolor*[21], *Alternaria alternata*[23], and *Trametes hirsuta* AK04[24]. Further, syringaldazine, benzoic acid, gallic acid, and vanillin could also enhance the laccase production from *Pleurotus sajor-caju* strain PS-2001[22]. Moreover, $CuSO_4$ and guaiacol were other inducers that could advance the production of laccase from *P. ostreatus*[20] and *Lentinus crinitus*[25]. Despite these advances, the response of *Perenniporia* sp. to various inducers remains underexplored mainly, requiring further investigation.

In this study, twelve fungal fruiting bodies were collected from decomposing logs in Tangkiling Hill Natural Park, Central Kalimantan, Indonesia. It resulted in the isolation of five fungal strains in pure culture. Among these, two isolates exhibited significant laccase activity during preliminary screening. *Perenniporia* sp. was selected for further study as it demonstrated the highest laccase production under nitrogen-limited conditions. Although other white-rot fungi were also isolated, their enzyme activity was comparatively lower than that of *Perenniporia* sp., so detailed optimization studies were conducted on this fungi. Therefore, this research aimed to investigate the effect of diverse inducers added into nitrogen-limited media on laccase production in *Perenniporia* sp. isolated from Tangkiling Hill Natural Park. By optimizing laccase production through selective inducers, this study contributes to the understanding of fungal enzyme regulation and provides a foundation for potential industrial and environmental applications.

2. Materials and methods

2.1 Location and sampling

The study site was located in Bukit Tangkiling Nature Park, Central Kalimantan Province, Indonesia. Fungal samples were collected from the site. The fungal fruit bodies were documented using a digital camera and then stored in containers. Data related to the fungal substrate and environmental conditions were recorded. The fungal species were then investigated through macroscopic observations, referring to fungal identification books.

2.2 Sample preparation

The fungal fruit bodies were cleaned and cut using a sterile scalpel to obtain the interior of the fungal mycelium. Small pieces of fungus (0.3 × 0.3 cm) were then sequentially soaked in 75% ethanol for 30 sec, 5.3% sodium hypochlorite for 5 min, 75% ethanol for 1 min, and sterile water for 5 min. They were then grown on sterilized PDA (Merck) containing chloramphenicol placed on Petri plates and incubated at 27 ± 2 °C. After that, the isolated fungal mycelium was sub-cultured onto sterilized PDA without chloramphenicol three times to obtain pure isolates. The growth rate of the pure isolate was then measured by assessing the diameter of the growing mycelium (in cm) daily until it filled the entire Petri plate.

The ITS region was assessed to extract and identify the genomic DNA of the fungal isolate in the ribosomal DNA through the PCR method. The DNA was separated by loading it onto 1% agarose gel and electrophoresed. The ITS-rDNA sequence was augmented with the forward and reverse primers in 20 µL of reaction. The reaction volume contained 20 ng/µL DNA template (2 µL) composed of 10 pmole/µL forward and reverse primers (each 1 µL), 10X Taq PCR buffer (2 µL), 2.5 mM dNTP mixture (1.6 µL), 2.5 U/µL KOMA-Taq (0.2 µL), and distilled water (HPLC grade) (13.2 µL). The PCR amplification process began with initial denaturation at 95°C for 5 min. It was continued with 30 cycles consisting of denaturation, annealing, and extension at 95°C for 30 sec, 55°C for 2 min, and 68°C for 1.5 min, respectively. Finally, a final extension was performed at 68°C for 10 min, using the Applied Biosystems™ Veriti™ 60-well Thermal Cycler. Then, the separate PCR primers and dNTPs were detached from the PCR products with the Montage PCR Clean-Up Kit (Millipore). The cleansed PCR products were sequenced at Macrogen Inc. (Geumcheon-gu, Seoul, Republic of Korea).

Next, the isolated fungal DNA sequences were aligned using MEGA 11.0.10 software. The sequences were then compared to the GenBank data with the BLAST tools accessible on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/blast/blast.cgi). It was done to verify the most closely related sequences. Afterward, phylogenetic trees were built following the hierarchical clustering of the ITS-rDNA sequence alignments. It was done using the Maximum Likelihood technique with the Kimura 2-Parameter + Gamma Distribution model at 1000 iterations[26] in MEGA 11.0.10 software.

2.3 Screening of fungal isolate for laccase production

The pure fungal mycelia were cultured on Petri dishes comprising sterilized PDA and given 0.02% guaiacol (Sigma, USA). They were incubated at 27°C for 10 days, and reddish-brown zones formed around the fungal colonies were observed.

2.4 Influence of the incubation period on the laccase production

Five mycelial disks (5 mm diameter) from actively growing fungal mycelia on PDA plates were inoculated

into Erlenmeyer flasks (250 mL) comprising a sterilized nitrogen-limited medium (20 mL). 1 L of the medium contained Basal III medium (100 mL), 120 mM ammonium tartrate (50 mL), 0.1 M phosphate buffer (pH 4.5) (100 mL), and 20% D-glucose solution (10 mL)[13,27]. The Basal III medium was composed of potassium dihydrogen phosphate (2 g), magnesium sulfate heptahydrate (500 mg), thiamine hydrochloride (5 mg), calcium chloride dihydrate (150 mg), and a mineral solution (70 mL). Meanwhile, the mineral solution was made of magnesium sulfate heptahydrate (3 g), zinc sulfate heptahydrate (56.2 mg), boric acid (10 mg), sodium molybdate dihydrate (8.5 mg), nitrilotriacetate (1.4 g), manganese (II) sulfate pentahydrate (350 mg), ferrous sulfate heptahydrate (100 mg), potassium aluminum sulfate dodecahydrate (5.4 mg), copper(II) sulfate pentahydrate (10 mg), cobalt(II) sulfate heptahydrate (55.2 mg), sodium chloride (1 g), and calcium chloride (82 mg) in water (1 L). The mycelial cultures were incubated at 27°C for 3 weeks under static and dark conditions. The culture media were then collected every 3 days, and the laccase enzyme performance was evaluated periodically.

2.5 Influence of adding inducers on laccase production

Five mycelial disks from vigorously growing fungal mycelia on PDA plates were inoculated into the sterilized nitrogen-limited media (20 mL). The inoculation procedure followed the steps described earlier. After two days of culture, various inducers (300 µL) were poured into each medium. The inducers were $CuSO_4$ (1 mM)[20], syringaldazine (0.5 mM)[22], guaiacol (1 mM)[14], veratryl alcohol (100 mM)[27], and ethanol (868 mM)[19].

One-way analysis of variance (ANOVA) was performed to analyze laccase activity with different inducers and determine whether significant differences existed among them. Following ANOVA, Tukey's post-hoc test was applied to identify specific pairwise differences between inducers. A significance level of $p < 0.05$ was considered statistically significant. All statistical analyses were performed using SPSS (version 29.0.2.0).

2.6 Laccase Performance

Laccase performance was examined by employing a syringaldazine substrate[28]. The reaction was done in a cuvette with a size of 3 mL. The cuvette contained 0.1 M sodium tartrate buffer (750 µL) with pH 5.3, 0.5 mM syringaldazine (200 µL), and an enzyme (1.8 mL). The reaction was observed from the increasing absorbance at 525 nm (ε_{525} = 65,000 $M^{-1}.cm^{-1}$) for 2 min. One katal (kat) of laccase performance means the number of enzymes prepared to oxidize 1 mol of tetramethoxy-azo-bis-methylenequinone per second.

3. Results

3.1 Fungal isolation and molecular identification

A fungal sample grown on a wood substrate was collected from Bukit Tangkiling Nature Park, Central Kalimantan. The sample was identified as a white rot fungus from the *Polyporaceae* family based on macroscopic observations. The fungal fruit body appeared to be directly attached to the wood substrate, lacking a stalk and exhibiting gregarious colonies. It had a hard texture, a brownish-black surface, and white edges and bottom (Fig. 1(a)). Additionally, the fungal fruit body, with the sample code CS23062, was isolated using the surface sterilization method, and the fungal mycelia were successfully cultured on PDA plates (Fig. 1(b)).

Figure 2 shows the growth curve of the CS23062 isolates in the radial direction. The growth of the isolate exhibited distinct phases. A two-day lag phase occurred, during which the inoculum adapted to the medium conditions. Following this, the fungal mycelium entered the log phase after three days of inoculation, characterized by rapid growth. During this phase, the growth curve formed a straight line, and the mycelium diameter increased to approximately 89.5 ± 0.71 mm from the third to the tenth day. The growth rate of the mycelium in the radial direction (Kr) was determined based on the slope of the straight line on the growth curve[29]. The Kr value for the CS23062 isolate was determined to be 9.28 mm/day, classifying it as a moderately growing fungus. Compared to other fungi, its growth rate is slower than *Trametes hirsuta* (14.17 mm/day) and *Marasmius* sp. (20.68 mm/day)[30] but faster than *Fomitopsis betulina* 2366 (8.00 mm/day)[31] and *Phellinus* sp. (5.92 mm/day)[32] when cultured on PDA medium. These findings suggest that while CS23062 exhibits moderate growth, its rate is within the expected range for basidiomycete fungi, mainly white rot species.

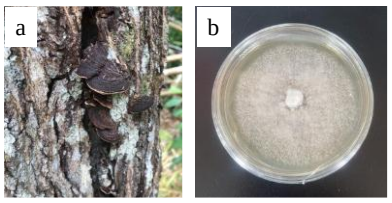

Fig. 1: (a) A fungal sample on the wood substrate (b) The fungal mycelia growth on the PDA plate.

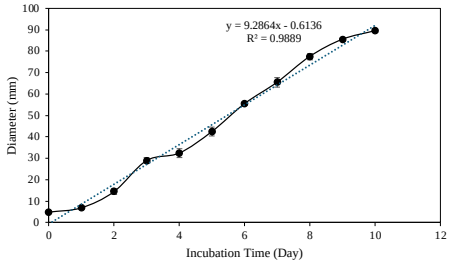

Fig. 2: The growth curve of the fungal isolate in the radial direction.

Further, the relatively slower growth of CS23062 compared to several white rot fungi could be attributed to its physiological adaptation to specific ecological niches. Environmental factors such as temperature and humidity also significantly affect fungal growth rates, underscoring the complex interplay between environmental variables and fungal physiology[33]. Additionally, genetic differences in the regulatory mechanisms governing mycelial expansion[34] may contribute to its moderate growth classification.

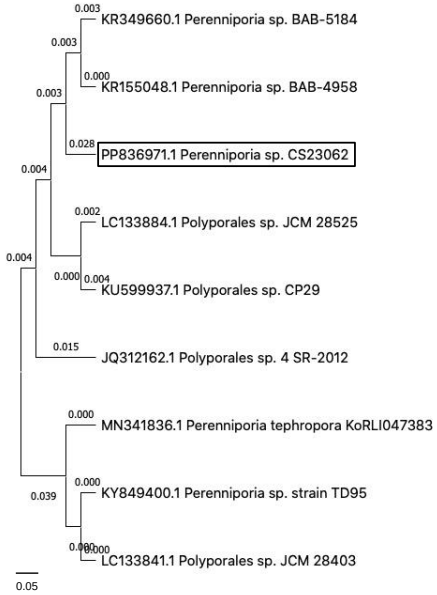

Fig. 3: Phylogenetic tree of the ITS-rDNA region showing the phylogenetic position of the fungal isolate in comparison with available ITS1-5.8S-ITS2 rDNA sequence data of other fungal species available in GenBank.

The CS23062 isolate was then molecularly identified using its ITS-rDNA sequence through the PCR technique. The analysis revealed an ITS-rDNA region with a sequence length of 621 bp. This ITS-rDNA sequence has been registered in the GenBank database under accession code PP836971.1. The ITS-rDNA sequence was then used to determine the phylogenetic placement of the isolate, which was related to *Perenniporia* sp. (Fig. 3).

3.2 Screening of the fungal isolate for laccase production

The CS23062 isolate was cultured on PDA supplemented with 0.02% guaiacol to screen for laccase performance. Guaiacol is a compound used for the easy and rapid detection of laccase performance. Isolates capable of producing laccase will form a reddish-brown zone around their colonies. This reddish-brown color indicates the presence of laccase performance, which catalyzes the oxidative polymerization of guaiacol into quinones[35]. The screening results showed that the CS23062 isolate produced laccase performance as a reddish-brown zone around the fungal colony was formed (Fig. 4).

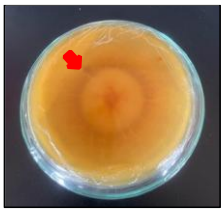

Fig. 4: The bottom views of fungal mycelium in the PDA plate supplemented with 0.02% guaiacol after 10 days of incubation isolate.

3.3 Influence of the incubation period on the laccase production

The influence of the incubation period on the laccase production by the CS23062 isolate was evaluated. The results revealed that the laccase production occurred maximum on the 12th day of culture (Fig. 5), where the highest laccase performance achieved about 1.32 nkat/mL. Nevertheless, the laccase performance of this isolate was relatively low compared to other polypore fungi. Therefore, it was deemed necessary to induce higher laccase performance in the CS23062 isolate using appropriate inducers.

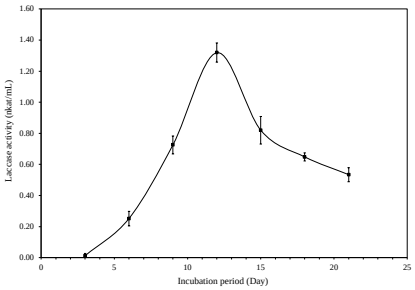

Fig. 5: Influence of incubation period on laccase production.

3.4 Influence of the addition of the inducer on the laccase production

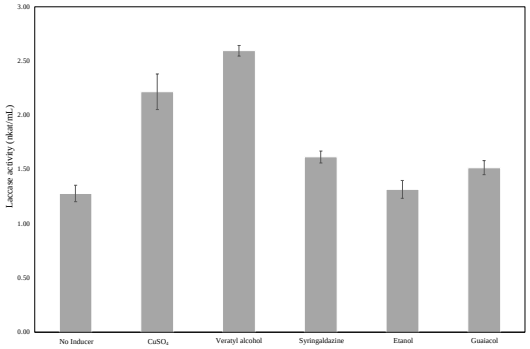

Fig. 6: Effect of inducers on laccase production.

Figure 6 illustrates the influence of adding different inducers on laccase production in the CS23062 isolate. It demonstrates that adding various inducers could improve the laccase production. The order for the laccase production based on the inducers was veratryl alcohol (2.59 nkat/mL) > $CuSO_4$ (2.21 nkat/mL) > syringaldazine (1.62 nkat/mL) > guaiacol (1.52 nkat/mL) > ethanol (1.36 nkat/mL). This indicates that the nitrogen-limited medium

supplemented with veratryl alcohol was the most effective for inducing laccase performance in the CS23062 isolate.

4. Discussion

In this research, veratryl alcohol was identified as the most effective inducer for laccase production in *Perenniporia* sp., possibly due to its dual role as a redox mediator and a lignin-derived signaling molecule. Veratryl alcohol, also known as 3,4-dimethoxybenzyl alcohol, is a secondary metabolite from the aromatic alcohol group. It is synthesized de novo from glucose by basidiomycete fungi. It is believed to serve as a physiological cofactor for the enzyme lignin peroxidase (LiP), which is crucial in lignin depolymerization. Its primary role is to act as a redox mediator for lignin peroxidase, protecting the enzyme during the redox cycle involving metabolically produced H_2O_2[36].

Beyond its role in LiP activity, veratryl alcohol has also been reported to function as a laccase inducer in both basidiomycete and ascomycete fungi[21,23,24]. It is a characteristic that aligns with its ability to mimic natural lignin degradation products. Its structural similarity to these compounds may trigger laccase gene expression, simulating the environmental cues fungi encounter during wood decomposition[37]. Besides, veratryl alcohol participates in redox cycling, which can induce oxidative stress—an essential factor in the upregulation of laccase as part of the fungal adaptive response[38,39]. Furthermore, its interaction with transcriptional regulators may enhance the expression of laccase-related genes, further explaining its effectiveness[38].

Previous studies have consistently demonstrated its role as a potent enhancer of ligninolytic enzyme activity, reinforcing its significance in fungal enzyme regulation[37,38,40]. Statistical analysis in this study revealed significant differences in laccase production among the inducers ($p < 0.05$, one-way ANOVA). Veratryl alcohol significantly increased laccase production from 1.23 nkat/mL to 2.59 nkat/mL, representing a 110.57% increase compared to the control (without inducer). Tukey's post-hoc test then confirmed that veratryl alcohol induced significantly higher laccase production than $CuSO_4$ ($p < 0.05$) and all other organic inducers. These findings highlight its potency as a laccase enhancer, further reinforcing its crucial role in fungal enzyme regulation.

Furthermore, the second most effective inducer after veratryl alcohol was $CuSO_4$. This compound is commonly used to enhance laccase enzyme production in fungi because the Cu^{2+} ion is a crucial component of the laccase enzyme, serving as its active central atom. The attendance of Cu^{2+} ions is essential for the catalytic synthesis of the laccase protein. Kumar et al. (2011) noted that Cu^{2+} ions can increase the transcription rate of the laccase-encoding gene[20]. Additionally, several laccase-encoding genes can be expressed constitutively in response to nitrogen deficiency or the presence of Cu^{2+} ions. Baldrian et al. (2003) conveyed that the promoter region of laccase-encoding genes contains recognition sites specific for metals such as Cu[17]. Palmieri et al. (2001) demonstrated that the laccase gene in *Pleurotus sp.* can be transcriptionally induced by Cu^{2+} ions, which are thought to interact with the promoter region of the laccase gene[41]. Furthermore, Santana et al. (2018) found that combining $CuSO_4$ with veratryl alcohol in the culture medium could also induce laccase production in *Lentinus crinitus*, where this combination stimulated laccase production by up to 100%[42].

Besides $CuSO_4$, organic inducers such as syringaldazine, guaiacol, and ethanol could also enhance laccase enzyme production in this research. Further, it has also been reported that these compounds could induce laccase production in numerous white rot fungi[19,20,22]. As a response to the introduction of these compounds, laccase-encoding genes in white rot fungi are distinctively managed[43]. This induction usually happens at the transcriptional level over interactions with DNA sequences or xenobiotic-responsive elements (XRE) located in the promoter area of the laccase gene. Giardina et al. (2010) exposed that these DNA sequences promote the transcription of the laccase gene in the presence of organic compounds during the interaction with regulatory proteins[44]. Hence, the laccase production serves as a defense system for chemical stress induced by organic compounds given to the fungal culture medium. The ability of these compounds to induce laccase production suggests that fungi possess a regulatory system. This system responds to environmental chemical stress by activating laccase as a protective mechanism. This mechanism is particularly relevant for fungi involved in the degradation of complex organic compounds in nature. It also has potential environmental applications where laccase is required for pollutant breakdown.

Laccase is widely recognized for its potential in bioremediation, especially in the degradation of environmental pollutants such as textile dyes, pesticides, and phenolic compounds commonly found in industrial wastewater[45–47]. The study's findings on the efficacy of veratryl alcohol as a laccase inducer provide a valuable foundation for optimizing large-scale enzyme production for environmental applications. Further, scaling up enzyme production would enable cost-effective, eco-friendly treatments and reduce chemical reliance and toxic byproducts. This approach aligns with the growing demand for sustainable wastewater treatment solutions that mitigate environmental pollution without introducing secondary contaminants.

Despite these findings, this study had several limitations. The selection of inducers was based on previous literature and their relevance to the targeted conditions. It allowed for an in-depth analysis of specific factors but restricted the generalizability of the results. Besides, this study only provided preliminary insights into the laccase induction in *Perenniporia* sp. with common

inducers, leaving the responses to other unexamined inducers unexplored. Additionally, this study was conducted under controlled experimental conditions without considering various environmental factors that could influence enzyme production. Variables such as pH, temperature, carbon and nitrogen sources, and the presence of additional cofactors or inhibitors may significantly alter the effectiveness of inducers and overall enzyme yield. Moreover, the absence of these considerations limits the findings' applicability to real-world scenarios, particularly for large-scale industrial or environmental applications.

Accordingly, future studies should explore a broader range of inducers and environmental conditions to develop a more comprehensive understanding of laccase enzyme regulation. Besides, investigating diverse inducer combinations, optimizing culture conditions, and integrating molecular techniques will be crucial for elucidating the complex mechanisms governing laccase induction. Additionally, transcriptomic and proteomic approaches can provide deeper insights into the genetic and biochemical pathways involved in laccase regulation, facilitating the development of more efficient enzyme production systems. These advancements will not only enhance the industrial and environmental applications of laccase but also help bridge the gap between laboratory findings and practical, real-world implementation.

5. Conclusions

A white rot fungus with the sample code CS23062 was successfully isolated from Bukit Tangkiling Nature Park, Central Kalimantan. Based on macroscopic characteristics and molecular identification, the CS23062 isolate was classified as *Perenniporia* sp. The isolate was cultured in a nitrogen-limited medium with various inducers to evaluate their effect on laccase production. The results demonstrated that veratryl alcohol was the most effective inducer for enhancing laccase production in *Perenniporia* sp. CS23062, showing the highest enzyme activity among the tested inducers. However, since this study was conducted under specific culture conditions, further research is required to optimize laccase production by exploring diverse media compositions and environmental factors.

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