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The First Evidence of Potential Antibacterial Activity of Laccase Enzyme from Indonesian White Rot Fungi against Pathogenic Bacteria

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ABSTRACT

The antibacterial agent can be extracted from plants, animals, and microorganisms such as fungi. The potential antibacterial activity of laccase derived from fungi remains limited in current reports. This study aimed to investigate the characteristics of laccase from Indonesian white rot fungi (WRF) and explore its potential as an antibacterial agent. The laccases were produced by *Trametes hirsuta* D7, *Trametes hirsuta* EDN 082, *Leiotrametes menziesii* BRB 73, and *Lentinus sajor-caju* BRB 12 using oil palm empty fruit bunch as a substrate. The results showed that the Indonesian WRF tested produced brownish-yellow laccase. FTIR analysis demonstrated similar peak patterns but distinct absorption intensities among the laccases. *Trametes hirsuta* D7 gained 0.044 U/ml of the greatest laccase activity. Laccase, with minimal activity of 0.001 U/ml–0.026 U/ml, suppressed the propagation of *Propionibacterium acnes* and *Staphylococcus aureus. Escherichia coli* and *Pseudomonas aeruginosa* could be inhibited by the laccase with a minimum activity of 0.002 U/ml–0.044 U/ml. However, *S. aureus* and *E. coli* showed the Minimum Bactericidal Concentration in the laccase activity range of 0.018 U/ml–0.308 U/ml. Gram-positive and Gram-negative bacteria grow more slowly when the laccase is present, supposed the laccase as a potential antibacterial agent.

1. Introduction

Infectious disease is still a health issue. Poor hygiene, which promotes the growth of pathogenic bacteria in the environment, is one of the causes of infectious diseases. Pathogenic bacteria are parasitic bacteria that cause disease in their hosts. Diarrhea, digestive tract infections, respiratory tract infections, bladder tract infections, skin lining infections that cause acne, and other diseases are caused by pathogenic bacteria (Hou *et al.* 2022). Pathogenic bacteria commonly found include *Escherichia coli, Salmonella enterica, Propionibacterium acnes, Citrobacter rodentium, Pseudomonas aeruginosa, Listeria monocytogenes*, and *Staphylococcus aureus* (Baumler and Sperandio 2016; McLaughlin *et al.* 2019; Widowati *et al.* 2021). The spread of pathogenic bacteria can be slowed by inhibiting their growth with antibacterial compounds such as antibiotics. However, the current overuse of antibiotics causes issues such as the emergence of multidrug-resistant organisms. Antibacterial compounds are not only used in the medical and pharmaceutical fields but also in other industries such as food, agriculture, and cosmetics. It is typically used as a preservative to inhibit the development of microorganisms in goods that could compromise product quality (Mahmud and Khan 2018).

Antibiotic alternatives include the use of extracts of natural ingredients from plants, animals, or microorganisms that contain active compounds such as alkaloid compounds (AlSheikh *et al.* 2020; Stan *et al.* 2021). Groups of enzymes that include protease, lipase, amylase, cellulase, peroxidase, trypsin, lysozyme, and laccase, in addition to extracts of natural components, exhibit antibacterial activities (Charlotte *et al.* 2006; Aruwa *et al.* 2022). Laccase is a versatile enzyme that can degrade xenobiotic compounds, transform antibiotics and

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steroids, detoxify water, delignify pulp, and degrade wastewater dyes (Becker *et al.* 2016; Anita *et al.* 2020; Ramadhan *et al.* 2021; Yanto *et al.* 2021). Laccase is extensively present in higher plants (Dana *et al.* 2017), insects (Janusz *et al.* 2020), bacteria, and fungi (Bertrand *et al.* 2013). Laccases generated by microorganisms such as bacteria and fungi are easier to get since the enzyme is released outside the cell. Laccase generated by fungi has a higher redox potential than laccase produced by bacteria. The redox potential value is connected to the ability of these enzymes to digest high-molecular-weight substrates (Janusz *et al.* 2020).

Indonesia is known as a country with mega biodiversity for its flora, fauna, and microorganisms. The diversity of fungi in tropical rainforests ranks second after insects. Fungi are often found in the rainy season on decayed wood, litter, or as parasites in living plants (Khayati and Warsito 2016). Basidiomycota is a fungus with basidiocarps that grows in various shapes, colors, and sizes. White-rot fungi (WRF) are Basidiomycota, which colonize wood in nature and preferentially break down lignin to generate white rotting. Fungi cause three forms of wood decay: white rot, brown rot, and soft rot. These fungi are classified based on the pattern of decay on wood (Godell *et al.* 2008). WRF are the best lignin degraders. Their capacity to digest complex and resistant organic compounds makes them appealing microorganisms for the bioremediation of organically polluted soil as well as the decolorization of wastewater from the textile industry (Koyani *et al.* 2014; Yanto *et al.* 2021; Anita *et al.* 2022).

A previous study has shown that *T. hirsuta* D7 successfully decomposes chrysene, benzo[a]pyrene, and phenanthrene (Hidayat and Yanto 2018). *Trametes hirsuta* EDN 082, *Leiotrametes menziesii* BRB 73, and *Lentinus sajor-caju* BRB 12 proved a good capability to remove the colored textile dyes of anthraquinone, monoazo, and diazo (Anita *et al.* 2022; Nurhayat *et al.* 2022). Charlotte *et al.* (2006) discovered that laccase generated by the fungus *Myceliophtera thermophila* and *Polyporus pinisitus* with activity levels ranging from 0.1 to 5 mg/L could suppress the propagation of Gram-positive bacteria *Staphylococcus epidermidis* and Gram-negative bacteria *Pseudomonas aeruginosa*. Laccase from *M. thermophila* that was immobilized onto bacterial nanocellulose can inhibit the growth of Gram-positive bacteria *Staphylococcus aureus* and Gram-negative bacteria *Escherichia coli* as much as 92% and 26%, respectively (Sampaio *et al.* 2016). Verma *et al.* (2019) also reported that both crude and purified laccase enzymes produced from the bacteria *Pseudomonas putida* LUA15.1 inhibited the growth of fungal plant and bacterial pathogens. However, the antibacterial property of the laccase from white rot fungi has never been examined.

The purpose of this research is to characterize the laccase produced by white rot fungi *Trametes hirsuta* D7, *Trametes hirsuta* EDN 082, *Leiotrametes menziesii* BRB 73, and *Lentinus sajor-caju* BRB 12, and to evaluate its antibacterial activity against various pathogenic bacteria. This research used pathogenic bacteria such as *Staphylococcus aureus, Propionibacterium acnes, Pseudomonas aeruginosa*, and *Escherichia coli*. The antibacterial activity was determined using diffusion and dilution methods to obtain the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of laccase against pathogenic bacteria.

2. Materials and Methods

2.1. Substrate, Microbes, and Chemical Components

OPEFB (Oil Palm Empty Fruit Bunch) was collected from an oil palm farm in Cikasungka, West Java, Indonesia., Indonesia. *Trametes hirsuta* D7 (NCBI GenBank, accession No. KX444204) was previously isolated from a peat swamp forest region in Riau, Indonesia. *Trametes hirsuta* EDN 082 (NCBI GenBank, accession No. MT476912) was isolated from Taman Eden 100, Toba Samosir, North Sumatra, Indonesia. *Leiotrametes menziesii* BRB 73 (NCBI GenBank, accession No. MT804553) and *Lentinus sajor-caju* BRB 12 (NCBI GenBank, accession No. OR050821) were isolated from Berbak-Sembilang National Park, Jambi, and South Sumatra, Indonesia. Isolates of *Pseudomonas aeruginosa* ATCC 15442 and *Escherichia coli* ATCC 8739 were obtained from the IPBCC (IPB Culture Collection). *Staphylococcus aureus* ATCC 29213 and *Propionibacterium acnes* ATCC 27853 were purchased from the Indonesian retail market. Malt extract, potato dextrose agar (PDA), peptone, sodium acetate, glucose, ammonium sulfate ($(NH_4)_2SO_4$), and $CuSO_4$ were provided from Merck (Germany). Wako (Japan) supplied the acetic acid. Himedia (India) provided the nutrient agar (NA), nutrient broth (NB), Bradford reagent, and Bovine Serum Albumin (BSA), while Sigma Aldrich provided the 2,2-azino-bis-[3-ethyl benzothiazoline-6-sulphonic acid] (ABTS).

2.2. Fungal Cultivation and Production of Laccase

Fungal culture and laccase synthesis were carried out using the technique described by Ningsih *et al.* (2020), with modifications to the weight of the substrate employed. *Trametes hirsuta* D7, *Trametes hirsuta* EDN 082, *Leiotrametes menziesii* BRB 73, and *Lentinus sajor-caju* BRB 12 were separately cultivated on a PDA medium (39 g/L) and culture for 7 d at room temperature (27±3°C). Laccase was produced via solid-state fermentation using 5 g of OPEFB fiber in 100 ml of Erlenmeyer. The OPEFB substrate was then treated with up to 10 ml of malt extract-glucose-peptone (MGP) medium that consisted of 20 g/L malt extract, 20 g/L glucose, 1 g/L peptone, and 2 mM $CuSO_4$ to increase its moisture content to 60% and induce laccase synthesis, respectively. After sterilizing the mixture for 15 min at 121°C, they were cooled to room temperature. Six plugs (5 mm) of the PDA fungal colony were injected into the sterilized substrate and cultured for 10 d at room temperature.

2.3. Crude Laccase Extraction

Crude laccase extraction was carried out according to Anita *et al.* (2020). After the incubation period, the fermented solid substrates were fully extracted in a homogenizer at a speed of 10,000 rpm for 10 min in a cool environment with 15 ml of 0.1 M acetate buffer, pH 4.5. The mixtures were then filtered through filter fabric. The filtrates were centrifuged at 4°C, 8,000 rpm for 20 min and the supernatant was treated with $(NH_4)_2SO_4$ to produce a 40-60% (w/v) saturated solution. The solution was mixed for 1 h before being centrifuged at 4°C, 8,000 rpm for 20 min. After that, 15 ml of 0.1 M acetate buffer, pH 4.5 was added to the pellet. The crude laccases were then kept at –20°C. For concentrated enzyme, the enzyme solution was placed in an ultrafiltration membrane 10 kDa (YM-10 Amicon, USA) and centrifuged at 4°C, 10,000 rpm for 10 min (Anita *et al.* 2022). The solution retained on the ultrafiltration membrane is the concentrated crude enzyme and is stored at –20°C.

2.4. Laccase Characterization

Laccase color, functional groups, enzyme activity, protein content, and enzyme-specific activity were characterized. The colors of laccase analysis were conducted according to Bahanawan *et al.* (2019). The color of the enzymes produced was compared to commercial enzyme color and the absorbance of both colors was measured using a microplate reader (TECAN Infinite 200 Pro, Switzerland) at 200–500 nm. Color values were also measured using a Konica Minolta CR-10 Plus colorimeter with a D65 lighting source specification, a photodiode array sensor, and a 10° observer standard. Color value (E*) is a quantitative description of color. The color analysis used the CIE-Lab method. The color value was calculated using Eq. 1. (Bahanawan *et al.* 2019).

$$E^* = \sqrt{(L^*)^2 + (a^*)^2 + (b^*)^2} \qquad (1)$$

Where:
E * = color value
L * = brightness
a * = redness, and
b * = yellowness

The functional groups of the crude laccase were analyzed based on the procedure used by Samui and Sahu (2018) and Yanto *et al.* (2021) using Fourier Transform Infrared (FTIR) (Perkin-Elmer) at 400–4,000 nm and 32 scans. The bands were shown in baseline mode. Laccase enzyme activities were determined using the procedure used by Anita *et al.* (2020). The spectrophotometric method was employed to evaluate the activity of laccase. This was achieved by monitoring the oxidation of 1 mM 2,2-azino-bis-[3-ethyl benzothiazoline-6-sulphonic acid] (ABTS) in 0.05 M acetate buffers at a pH of 4.5. The measurements were taken at a wavelength of 420 nm for a duration of 1 minute, while maintaining a room temperature environment. The test combination comprised of 100 µL of the sample, 400 µL of 0.1 M acetate buffer, and 500 µL of 2 mM ABTS. The unit of enzyme activity (U) was established as the quantity of enzyme necessary to catalyze the oxidation of 1 µmol of ABTS per minute. While protein content was determined using the Bradford technique (Bradford 1976). Protein concentrations were measured in milligrams per milliliter (mg/ml). The protein standard in this research was bovine serum albumin (BSA) (1 mg/ml). The specific activity of the enzyme was calculated using Eq. 2. (Ningsih *et al.* 2020).

$$\text{Enzyme specific activity}\left(\frac{U}{mg}\right) = \frac{\text{Enzyme activity}\left(\frac{U}{ml}\right)}{\text{Protein content}\left(\frac{mg}{ml}\right)} \qquad (2)$$

2.5. Antibacterial Activity Test

A bacterial culture with a 10^8 CFU/ml cell count was used for the antibacterial activity assay. The bacterial culture was added to the sterile NA medium that had not yet solidified (medium temperature, 40°C) in amounts up to 1% (v/v) and carefully mixed. Then the media containing the bacterial culture were poured into the sterilized petri dish and allowed to set. Furthermore, on the solid medium, wells were made using a cork borer (Ø5 mm). 20 µl of Laccase enzyme samples (laccase from *T. hirsuta* D7, *T. hirsuta* EDN 082, *Leiotrametes menziesii* BRB 73, and *Lentinus sajor-caju* BRB 12) were added separately to each well, and chloramphenicol (CAP) 30 µg/ml used as a positive control. The plates were placed in an incubator for 24 h at 35±2°C. By measuring the clear area formed surrounding the hole, the inhibition zone in the medium was estimated (Modarresi-Chahardehi *et al.* 2012). Antibacterial inhibition zone activity was classified into categories: weak (less than 5 mm), average (5–10 mm), strong (10–20 mm), and extremely strong (over 20–30 mm) (Rahayu *et al.* 2021).

2.6. Analysis of The Minimum Inhibitory Concentration (MIC)

The MIC was obtained using the liquid dilution technique according to Modarresi-Chahardehi *et al.* (2012) with modification in the dilution series. The enzymes and sterile NB media were diluted in the following ratios: 2:0, 1.6:0.4, 1.2:0.8, 0.8:1.2, 0.4:1.6, 0.2:1.8, and 0.05:1.95 (ml). Each dilution of the enzyme sample was put into a microplate with as much as 500 µL and 25 µL of bacterial suspension (10^8 CFU/ml). As a positive control, 500 µL of NB liquid medium was added to 25 µL of bacterial suspension (10^8 CFU/ml) in a microplate. Cultures were incubated in an incubator for 24 h at 35±2°C. After 24 h, each sample's absorbance was measured by using a microplate reader at 660 nm. The lowest concentration that can inhibit bacteria was indicated by the absence of turbidity after incubation.

2.7. Analysis of The Minimum Bactericidal Concentration (MBC)

As much as 100 µL of bacterial isolates from the results of MIC incubation were spread into Nutrient Agar (NA) medium in sterilized dishes. Cultures were incubated in an incubator at 35±2°C for 24 h. The lowest concentration that can kill bacteria was shown by the absence of microbial growth on the agar media after incubation (Modarresi-Chahardehi *et al.* 2012).

2.8. Statistical Analysis

The assay was performed with three replicates. The data were subjected to analysis of variance (one-way ANOVA) and the means were compared using Tukey's test at the 5% level.

3. Results

3.1. Characterization of Laccase Enzyme

T. hirsuta D7, *T. hirsuta* EDN 082, *Leiotrametes menziesii* BRB 73, and *Lentinus sajor-caju* BRB 12 produces a brownish-yellow laccase. Meanwhile, the commercial laccase enzyme is white (Figure 1A). The laccase enzymes produced by the four fungi were more intense in color compared to commercial enzymes. The laccase enzyme from *T. hirsuta* D7 showed a higher color spectrum than the other enzymes (Figure 1B). In contrast, the color value of the laccase enzyme from *T. hirsuta* D7 is the lowest at 53.29±0.10 when compared to the color values of other laccase enzymes. The E values of laccase produced by the four fungi ranged between 53.29 to 62.29. While commercial laccase has a value of 91.93±1.15 (Table 1).

The laccase enzyme FTIR spectrum and identified bonds are shown in Figure 2 and summarized in Table 2. Characteristic bands of the commercial laccase were identified at 3298 cm^{-1} (O-H/N-H stretching), which corresponds to carbonyl groups and amide A structure; a peak at 2922 cm^{-1} (C-H/N-H stretching) attributed to CH_2 groups present in laccase protein; a peak at 1642 cm^{-1} (C = O / C - N stretching) from amide I with a β-sheet structure; a peak 1360 cm^{-1} (N-H bending/C-N stretching) from amide III bands, a peak at 1015 cm^{-1} (C-N/C–O–C) characteristic of protein in laccase; and a peak at 572 cm^{-1} (C = O bending) from amide V.

Laccase from *T. hirsuta* D7 and *T. hirsuta* EDN 082 showed the same peak. These laccases contained peaks that were not present in commercial ones, particularly around 3033/3061 to 3179 cm^{-1} (N-H stretching) from amide B and 1553/1555 cm^{-1} (N-H

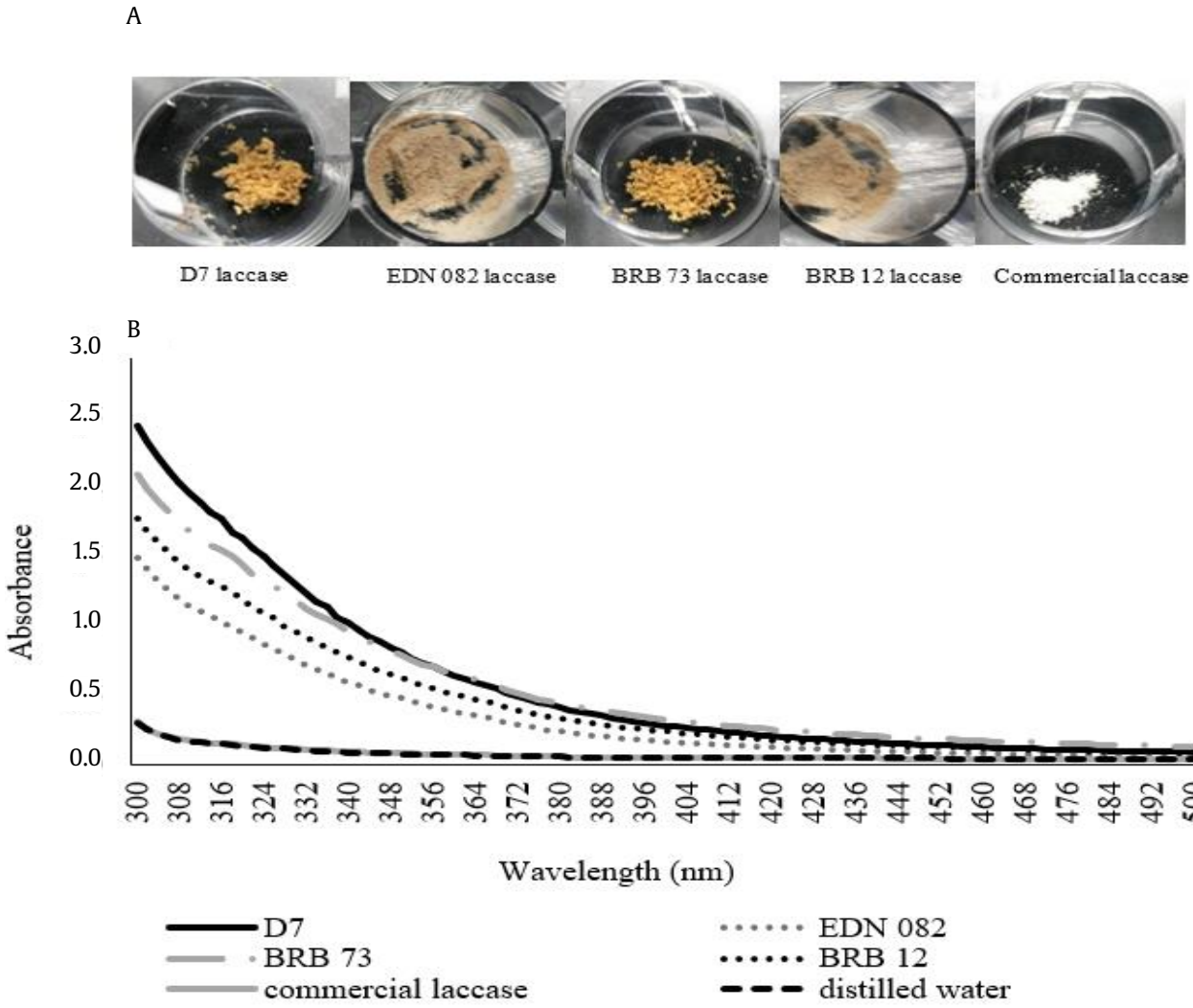

Figure 1. The color (A) and the UV-Vis spectrum (B) of the laccase enzyme

Table 1. Color value of laccase enzyme

Laccase enzyme	L (brightness)	a (redness)	b (yellowness)	E (color value)
D7	52.13±0.45[a]	10.13±0.21[b]	19.60±0.35[b]	53.29±0.47[a]
EDN 082	59.27±1.16[b]	10.00±0.17[b]	24.80±0.56[c]	60.31±1.16[b]
BRB 73	54.13±0.45[a]	10.03±0.15[b]	19.60±0.26[b]	55.23±0.46[a]
BRB 12	61.27±1.16[b]	10.07±0.15[b]	24.67±0.67[c]	62.29±1.15[b]
Commercial	91.90±0.10[c]	-0.43±0.06[a]	5.40±0.00[a]	91.92±0.09[c]

The mean value followed by the same letter is not significantly different according to Tukey (HSD) test at the 0.05 significance level

bending/C-N stretching) from the amide II band. However, some peaks were identical to those of commercial laccases, especially peaks at 1062/1068 cm^{-1} as the characteristic of protein in laccase and 608/609 cm^{-1} from amide V bands.

The peaks of BRB 12 and BRB 73 laccases show a similar pattern. *Leiotrametes menziesii* BRB 73 and *Lentinus sajor-caju* BRB 12 laccases also contained peaks that did not appear in commercial laccases. The peaks are at 3028/3030 cm^{-1} to 3193/3197 cm^{-1} from the amide B and 1553 cm^{-1} from the amide II band. BRB 12 and BRB 73 laccases have peaks at 1276/1280 cm^{-1} to 1403/1404 cm^{-1}, 1069/1094 cm^{-1}, and 609/610 cm^{-1} attributed to the amide III bands,

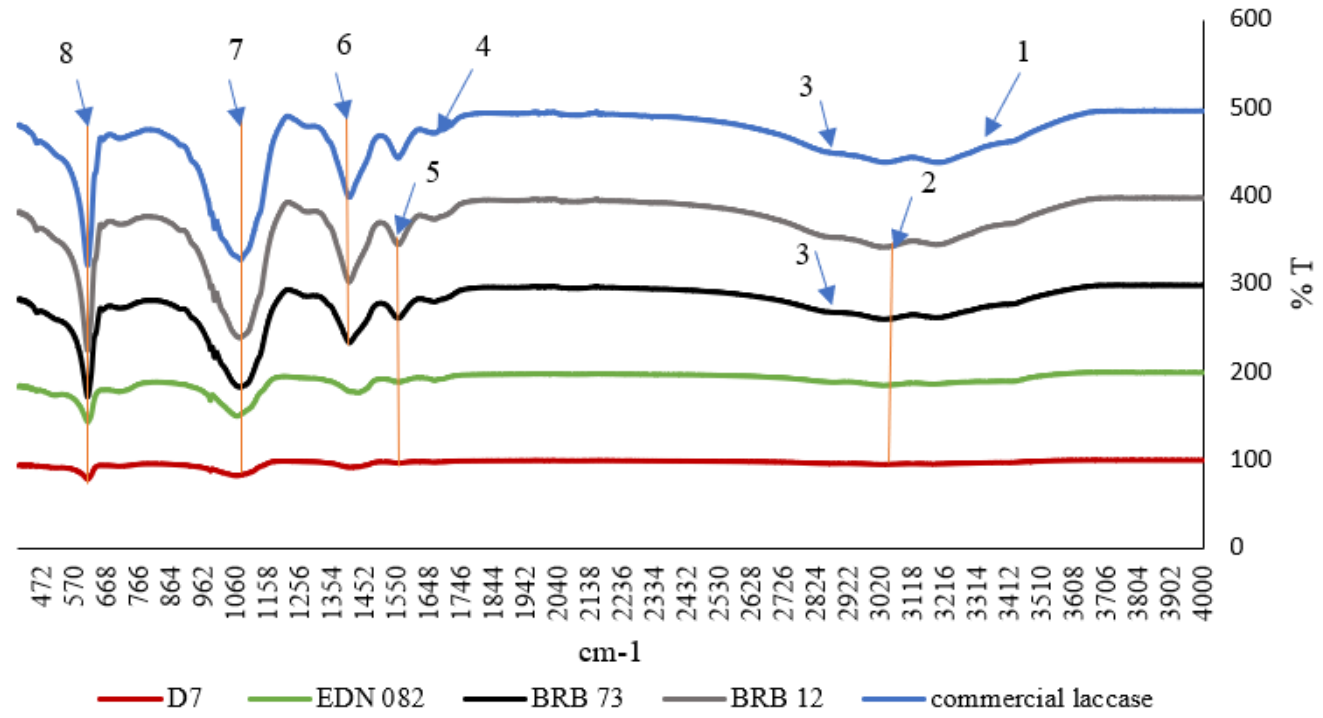

Figure 2. Laccase enzyme FTIR spectrum

Table 2. Functional groups of laccase enzyme FTIR spectrum

D7 laccase	EDN 082 laccase	BRB 12 laccase	BRB 73 laccase	Commercial laccase	Functional group assignment (references)	Class compound
Wave number (cm^{-1})						
-	-	-	-	3298	O-H stretching, N-H stretching	Carbonyl group, Amide A
3061	3179-3033	3193-3028	3197-3030	-	N-H stretching	Amide B
-	-	-	2851	2922	C-H stretching, N-H stretching	CH_2 group
-	-	-	-	1642	C=O stretching vibration, C-N stretching vibration	Amide I with a β-sheet structure
1555	1553	1553	1553	-	N-H bending,C-N stretching vibration	Amide II bands
-	-	1403-1280	1404-1276	1360	N-H bending, C-N stretching	Amide III bands
1062	1068	1069	1094	1015	C-N stretching, C–O–C stretching	Protein in laccase, Aromatic amine
608	609	609	610	572	C=O bending	Amide VI bands

characteristic of protein in laccases, and the amide VI band, respectively. The three peaks are identical to those of commercial laccases.

The maximum laccase enzyme activity was obtained in *Trametes hirsuta* D7, which was 0.044 U/mL. In contrast, the protein content of *T. hirsuta* D7 laccase was lower than that of other fungi. Due to this, the laccase from *T. hirsuta* D7 has the greatest specific enzyme activity compared to the other laccases. The ultrafiltration technique increased the activity of concentrated laccase enzymes up to 12 times. Table 3 describes the laccase enzyme in greater depth.

3.2. Antibacterial Activity

The width of the inhibitory area was used to evaluate the bacteria-static effect of the laccase enzyme (Table 4). The results showed that almost all of the inhibition zones for crude laccase enzyme from fungi were less than 7 mm in diameter. However, once the enzyme was concentrated, the width of the inhibitory zone over *S. aureus*, *P. acnes*, *P. aeruginosa*, and *E. coli* increased from 7 to 12 mm.

3.3. Minimum Inhibitory Concentration (MIC)

Table 5 shows the MIC values of the laccase enzyme against various bacterial species, indicating antibacterial activity. Laccase activity isolated from *T. hirsuta* D7 ranged from 0.001U/ml to 0.044 U/ml, whereas that of *T. hirsuta* EDN 082 ranged from 0.001 U/ml to 0.009 U/ml. Laccase activity from *Leiotrametes menziesii* BRB 73 and *Lentinus sajor-caju* BRB 12 was 0.002–0.028 U/ml and 0.001–0.01 U/ml, respectively. In general, minimal laccase enzyme concentrations of 0.001 to 0.015 U/ml suppressed *S. aureus* growth.

The concentration of laccase enzyme necessary to suppress the growth of *P. acnes* was higher, at 0.002–0.026 U/ml. The growth of *P. aeruginosa* could be suppressed with a minimal laccase concentration of 0.004–0.026 U/ml. Meanwhile, the lowest laccase enzyme concentration required to inhibit *E. coli* growth is 0.002–0.044 U/ml.

3.4. Minimum Bactericidal Concentration (MBC)

Table 6 shows the MBC value of the laccase enzymes. The MBC value for *S. aureus* was a laccase enzyme with activity between 0.018 U/ml and 0.112

Table 3. The characterization of laccase enzyme produced by *T. hirsuta* D7, *T. hirsuta* EDN 082, *Leiotrametes menziesii* BRB 73, and *Lentinus sajor-caju* BRB 12

Parameter	D7	EDN 082	BRB 73	BRB 12
Crude enzyme activity (U/ml)	0.044	0.009	0.028	0.010
Crude protein content (mg/ml)	18.36	22.67	22.34	21.10
Crude enzyme-specific activity (U/mg)	0.0020	0.0004	0.0013	0.0005
Concentrated enzyme activity (U/ml)	0.308	0.112	0.076	0.018
Concentrated protein content (mg/ml)	25.10	20.95	24.88	25.40
Concentrated enzyme-specific activity (U/mg)	0.012	0.005	0.003	0.0007

Table 4. Inhibition zone of laccase enzyme against *S. aureus, P. acnes, P. aeruginosa,* and *E. coli*

Laccase enzyme	Crude enzyme				Concentrated enzyme			
	Inhibition zone (mm)				Inhibition zone (mm)			
	S. aureus	*P. acnes*	*P. aeruginosa*	*E. coli*	*S. aureus*	*P. acnes*	*P. aeruginosa*	*E. coli*
D7	0.0±0.0^{a}	4.5±2.5^{b}	0.0±0.0^{a}	5.0±0.0^{a}	8.0±0.0^{ab}	7.0±0.0^{a}	10.0±0.0^{a}	7.7±0.6a
EDN 082	1.0±0.0^{a}	5.0±0.0^{b}	0.0±0.0^{a}	5.0±0.0^{a}	8.0±0.0^{ab}	9.0±0.0^{b}	10.0±0.0^{a}	7.5±0.5a
BRB 73	6.5±0.1^{c}	0.0±0.0^{a}	1.0±0.1^{a}	5.0±0.0^{a}	9.0±1.0^{b}	7.0±0.0^{a}	12.0±1.0^{b}	9.0±1.0^{ab}
BRB 12	4.5±0.2^{b}	0.0±0.0^{a}	0.0±0.0^{a}	5.0±0.0^{a}	7.0±0.0^{a}	7.5±0.5^{a}	10.0±0.0^{a}	7.7±0.6^{a}
Chloramphenicol (CAP)	12.3±1.3^{d}	13.0±0.0^{c}	19.3±1.2^{b}	11.0±1.7^{b}	12.3±1.3^{c}	13.0±0.0^{c}	19.3±1.2^{c}	11.0±1.7^{b}

The mean value followed by the same letter is not significantly different according to Tukey (HSD) test at the 0.05 significance level

Table 5. Minimum Inhibitory Concentration (MIC) of laccase enzyme against bacterial strain

Laccase	Gram type	Test organism	Minimal inhibitory concentration (MIC)		
			Enzyme activity (U/ml)	Protein content (mg/ml)	Specific activity (U/mg)
T. hirsuta D7	Gram (+)	*S. aureus*	0.015	3.67	0.0040
		P. acnes	0.026	7.35	0.0040
	Gram (-)	*P. aeruginosa*	0.015	3.67	0.0040
		E. coli	0.026	7.35	0.0040
T. hirsuta EDN 082	Gram (+)	*S. aureus*	0.001	4.53	0.0002
		P. acnes	0.004	9.07	0.0004
	Gram (-)	*P. aeruginosa*	0.004	9.07	0.0004
		E. coli	0.006	13.60	0.0004

Table 5. Continued

Laccase	Gram type	Test organism	Minimal inhibitory concentration (MIC)		
			Enzyme activity (U/ml)	Protein content (mg/ml)	Specific activity (U/mg)
Leiotrametes menziesii BRB 73	Gram (+)	*S. aureus*	0.003	4.47	0.0006
		P. acnes	0.006	8.94	0.0007
	Gram (-)	*P. aeruginosa*	0.014	13.96	0.0010
		E. coli	0.003	4.47	0.0006
Lentinus sajor-caju BRB 12	Gram (+)	*S. aureus*	0.002	4.22	0.0005
		P. acnes	0.002	8.44	0.0002
	Gram (-)	*P. aeruginosa*	0.008	13.19	0.0006
		E. coli	0.002	4.22	0.0005

Table 6. Minimum bactericidal concentration (MBC) of laccase enzyme against bacterial strain

Laccase	Gram type	Test organism	Minimum bactericidal concentration (MBC)		
			Enzyme activity (U/ml)	Protein content (mg/ml)	Specific activity (U/mg)
T. hirsuta D7	Gram (+)	*S. aureus*	0.044	18.36	0.002
		P. acnes	-	-	-
	Gram (-)	*P. aeruginosa*	-	-	-
		E. coli	0.308	25.10	0.012
T. hirsuta EDN 082	Gram (+)	*S. aureus*	0.112	20.95	0.005
		P. acnes	-	-	-
	Gram (-)	*P. aeruginosa*	-	-	-
		E. coli	0.112	20.95	0.005
Leiotrametes menziesii BRB 73	Gram (+)	*S. aureus*	0.076	24.88	0.003
		P. acnes	-	-	-
	Gram (-)	*P. aeruginosa*	-	-	-
		E. coli	0.076	24.88	0.003
Lentinus sajor-caju BRB 12	Gram (+)	*S. aureus*	0.018	25.40	0.001
		P. acnes	-	-	-
	Gram (-)	*P. aeruginosa*	-	-	-
		E. coli	0.018	25.40	0.001

U/ml. Meanwhile, greater laccase enzyme activities (0.018 to 0.308 U/ml) were required to kill *E. coli* bacteria completely. However, the laccase enzyme in the activity range of 0.018–0.308 U/ml could not kill *P. acnes* and *P. aeruginosa* bacteria.

4. Discussion

Four enzymes produced by four distinct microorganisms were investigated as antibacterial agents. Laccases are produced by *Trametes hirsuta* D7, *Trametes hirsuta* EDN 082, *Leiotrametes menziesii* BRB 73, and *Lentinus sajor-caju* BRB 12. The laccase enzymes produced by these different kinds of fungi have distinct properties. The laccases generated by all the fungi exhibited a brownish-yellow hue. This color contrasts with the commercial laccase enzyme from *Trametes versicolor*, which is white. The brown color appears because the extracted laccase enzyme has not been purified like commercial laccase; therefore, there are still numerous impurities that may be found in the crude laccase. Dhevagi *et al.* (2021) reported that the crude enzyme mixtures are complex, multielement, and consist of nonenzymatic proteins as well as secondary metabolites. The color of crude enzymes can also be caused by the residual fermentation medium used to cultivate enzyme-producing microorganisms. Crude enzymes from culture broth, including the growth media, organisms (whole or fragmented), and enzymes of interest (Robinson 2015). Zaccaria *et al.* (2019) used activated carbon to pre-treat crude enzyme extract to remove colors and inhibiting compounds. The UV-Vis spectra of crude laccase from four fungi were compared to the spectra of commercial enzymes and distilled water to evaluate the color intensity of the crude enzymes. Crude laccase from *T. hirsuta* D7 has the highest color

absorption compared to laccase from other fungi. Even the absorbance of commercial enzymes is comparable to distilled water's color absorption. A high absorbance value implies a higher quantity of substances, making the exhibited color darker (Neldawati *et al.* 2013). The color values (E) of the laccase enzyme generated by four white rot fungi were lower than those of commercial laccase, indicating that the laccase produced is less bright. In this study, the color values of laccase are related to lightness and yellowness values. Other studies have found a correlation between increased lightness (L) and yellowness (b) and higher E values, but not redness (a) levels (Bahanawan *et al.* 2019).

Enzymes are proteins that are composed of amino acids connected by one or more peptide bonds. Protein-peptide bonds are amide groups. Protein-peptide linkages are amide groups. In an IR spectrum between 4000 and 400 cm^{-1}, protein-containing samples show 9 unique vibrational absorption bands caused by various vibrational modes of the amide groups of proteins, notably amides A, B, and I-V (Bart 2007; Ji *et al.* 2020; Sadat and Joye 2020). The amide A, absorbing between 3500 and 3270 cm^{-1}, arises from N-H stretching vibration. While amide B absorbs weakly between 3100 and 3030 cm^{-1} (Bart 2007; Ji *et al.* 2020). Around 1600 and 1700 cm^{-1}, amide I vibrations absorb significantly and are particularly sensitive to the secondary protein structure. The amide II band absorbs at around 1550 cm^{-1} due to a combination of C-N stretching and N-H bending motions (Chatterley *et al.* 2022). The interaction of the N-H bending and the C-N stretching vibrations gives rise to the amide III bands between 1420 and 1200 cm^{-1} (Bart 2007; Samui and Sahu 2018). The amide IV and amide V areas are represented by absorption bands at 625–770 cm^{-1} and 640–800 cm^{-1}, respectively. The band of absorption at 537–606 cm^{-1} is associated with the amide VI regions because of the out-of-plane C = O bending (Darwish and Darwish 2022).

Laccase enzyme, as a protein, mostly exhibits peaks from nine distinct vibrational absorption bands in the IR spectrum. However, laccase's characteristic bands were identified at 1624 and 1690 cm^{-1} (amide I), 1420-1210 cm^{-1} (amide III), 1165–948 cm^{-1} (characteristic of protein in laccase), and at 800–500 cm^{-1} (amide V and VI) (Samui and Sahu 2018; Yanto *et al.* 2021). The peaks that were characterized as laccase were also seen in the laccase generated by the four fungi in this investigation. Laccase from *T. hirsuta* D7 and *T. hirsuta* EDN 082 showed the same peaks in the spectra at 155-153 cm^{-1} (amide II) and 609-608 cm^{-1} (amide VI). Meanwhile, the peaks laccase from *Leiotremetes menziesii* BRB 73 were more comparable to the laccase from *Lentinus sajor-caju* BRB 12. They shared the same peaks at 1404–1276 cm^{-1} (amide III) and 610–609 cm^{-1} (amide VI). Several peaks differed when the laccase IR spectra of the four fungi were compared to commercial *T. versicolor* laccase. The commercial laccase of *T. versicolor* has a peak at 1642 cm^{-1} (amide I), 1360 cm^{-1} (amide III), and 572 cm^{-1} (amide VI).

The differences in the peaks achieved could be because laccase was produced by different species of fungi, so the functional groups of the proteins tend to be slightly different. Nandiyanto *et al.* (2019) explained that the fingerprint area (600–1500 cm^{-1}) tends to be unique and distinct for any compound. However, the laccase produced by the four fungi shows a peak at 1094–1062 cm^{-1}, indicating the protein properties of the laccase enzyme.

Trametes hirsuta D7 produced the highest crude laccase enzyme activity with the lowest protein content compared to laccase from other species of fungi. As a result, *T. hirsuta* D7 laccase showed the greatest specific activity. Specific activity determines the purity of the enzymes in the mixture. It is the quantity of product generated by an enzyme in a specific period under specific circumstances per milligram of total proteins. The value increases when the amount of protein in an enzyme preparation decreases. At the same time, the reaction rate remains constant or may increase due to less interference or the elimination of inhibitors (Robinson 2015).

The crude laccase extract was then concentrated, and the laccase produced by *T. hirsuta* D7 enhanced its enzyme activity by up to 12-fold. *Trametes hirsuta* D7-specific laccase enzyme activity rises in proportion to enzyme activity. The findings of this study can be compared to those of earlier investigations. Laccase activity from *Aspergillus nidulans* increases 5.5-fold after the ultrafiltration process (Vivekanandan *et al.* 2014). Laccase activity after the ultrafiltration process compared to crude enzyme broth extracted from *Pleurotus sajor-caju* PS-2001 also increased 14-fold (Zaccaria *et al.* 2019). A 20-fold concentration of crude laccase by *Trametes*

versicolor was obtained using a UF membrane (10 kDa) (Antecka *et al.* 2019). After ultrafiltration, the crude enzyme laccase generated by *Pleurotus ostreatus* increases enzymatic activity by 6.6-fold. (Nguyen *et al.* 2020). Ultrafiltration membranes were shown to be effective in eliminating bigger particles from the medium and in concentrating certain enzymes (Zaccaria *et al.* 2019).

Laccase enzyme bactericidal activity was tested using *S. aureus, P. acnes, P. aeruginosa*, and *E. coli*. The clear zone generated surrounding the hole was used to determine the antibacterial action. The zone of bacterial growth inhibition was measured in millimeters. Crude laccase enzymes from *T. hirsuta* D7, *T. hirsuta* EDN 082, *Leiotrametes menziesii* BRB 73, and *Lentinus sajor-caju* BRB 12 created an inhibitory zone in the weak to moderate category. After the laccase enzyme was concentrated, it created an inhibitory zone ranging from moderate to strong. Li *et al.* (2019) also reported that the diameter of the inhibition zone of laccase-catalyzed chitosan–gallic acid derivative against *E. coli* and *S. aureus* was 11.36 and 12.65 mm, respectively, indicating the strong antibacterial activity of laccase. Rahayu *et al.* (2021) classified antibacterial inhibition zone activity into categories: weak (less than 5 mm), average (5–10 mm), strong (10–20 mm), and extremely strong (over 20–30 mm).

The diffusion approach was used in this study to detect the antibacterial properties of the laccase enzymes; however, it was unable to quantify the number of enzymes that inhibited or killed bacterial growth. As a result, the MIC of the laccase enzyme was measured to identify the lowest laccase concentration required to inhibit the growth of the bacterial strains, including *Staphylococcus aureus, Propionibacterium acnes* (Gram-positive bacteria) and *Pseudomonas aeruginosa, Escherichia coli* (Gram-negative bacteria). The crude extract laccase enzyme produced by the four fungi suppressed the propagation of pathogenic bacteria, with MIC values that ranged from 0.001 to 0.026 U/ml for Gram-positive bacteria and 0.002 to 0.044 U/ml for Gram-negative bacteria (Table 5). Li *et al.* (2019) also reported that *S. aureus* was slightly more sensitive to laccase-catalyzed chitosan–gallic acid derivative than *E. coli.* The activity of the laccase enzyme to suppress Gram-negative bacteria was greater than that to suppress Gram-positive bacteria. This is probably due to the more complex structure of the cell wall and outer membrane in Gram-negative bacteria which play a role in the mechanism of their resistance to exposure to antibacterial agents. Gram-negative bacteria have a wall and an outer complex membrane while having low levels of peptidoglycan, which contributes to their resilience (Sampaio *et al.* 2016; Besufekad *et al.* 2017).

Crude extract laccase enzyme exhibited poor bactericidal activity against pathogenic bacteria. Therefore, greater laccase enzyme activity from concentrated laccase is required to kill bacteria completely. MBC values ranging from 0.018 to 0.308 U/ml indicated their ability to kill *S. aureus* and *E. coli*. However, higher laccase enzyme activity was needed to kill *P. acnes* and *P. aeruginosa*. Many previous studies have found differences in laccase reactivity of the same or different types of fungi on various substrates. One of the elements influencing the differential in reactivity to the substrate is the laccase enzyme's molecular mass (Mansur *et al.* 2003). In general, the antibacterial process can be triggered for numerous reasons, including destroying or disrupting the molecules that constitute the bacterial wall, removing cell components, and carrying out processes that disturb the function of genetic material. Bioactive substances can disrupt the synthesis of RNA and DNA, causing harm to the resulting genetic material. Inhibition of bacterial growth can also occur due to the inhibition of enzymes by microbes, resulting in the destabilization of the cytoplasmic membrane found in these bacteria (Aruwa *et al.* 2022). The antibacterial activity of laccases is attributed to their method of action, which involves an electrochemical process that enables them to enter the cell walls of microbes. This penetration leads to the leaking of important metabolites and the physical disruption of crucial cell activities (Sampaio *et al.* 2016). Apart from the type of bacteria, the effectiveness of the antibacterial compounds of the enzymes is greatly influenced by the characteristics of the enzymes, such as their molecular mass and subunits (Mansur *et al.* 2003).

In conclusion, the four crude laccase enzymes produced by the Indonesian white rot fungi *Trametes hirsuta* D7, *Trametes hirsuta* EDN 082, *Leiotrametes menziesii* BRB 73, and *Lentinus sajor-caju* BRB 12 exhibited inhibitory effects on both Gram-positive and Gram-negative bacteria. The effectiveness of inhibition was found to be correlated with the

laccase activities employed in the experiment. The activity of the concentrated laccase enzyme increased by up to 12-fold, demonstrating its capability to eradicate *Propionibacterium acnes* and *Pseudomonas aeruginosa*. These significant findings contribute to the exploration of native Indonesian white rot fungi as potential sources of antibacterial enzymes.

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