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

ABSTRACT

The antibacterial agent can be isolated from plants, animals, and microorganisms such as fungi. The report related to potential activity of laccase produced from fungi as an antibacterial is still limited. report. In this study, the identification of the laccase characteristics from Indonesian white rot fungi (WRF), as well as its potential as an antibacterial agent, were explored. The laccaseswereproduced by *Trametes hirsuta* D7, *Trametes hirsuta* EDN 084, *Leiotrames menziesii* BRB 73, and *Lentinus* sp. BRB 12 uses oil palm empty fruit bunch as a substrate. The results showed that the Indonesian WRF tested produced brownish-yellow laccase. FTIR analysis revealed the same peak pattern but distinct absorption intensities between the laccase. *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.

Keywords: Antibacterial activity, Laccase enzyme, *Trametes hirsuta,Leiotrametes menziesii, Lentinus* sp.

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, Citrobacterrodentium*, *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 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 andWarsito 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, brownrot, and soft rot. These fungi are classified based on the pattern of decay on wood (Godell*et al*. 2008). WRF are thebest 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). *Trameteshirsuta* EDN 082, *Leiotrametesmenziesii* BRB 73, and *Lentinus* sp. BRB 12 proved a good capability to remove the colored textile dyes of anthraquinone, monoazo, and diazo (Anita *et al*. 2022; Nurhayat e*t al*. 2022). Charlotte *et al.* (2006) discovered that laccase generated by the fungus *Myceliophterathermophila* and *Polyporuspinisitus* 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*. However, the antibacterial property of

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the laccasefrom white rot fungi has never been examined, and only a few research have specifically described its usage as an antibacterial agent.

Therefore, this research purposes to characterize the laccase generated by white rot fungi *Trameteshirsuta* D7, *Trameteshirsuta* EDN 082, *Leiotrametesmenziesii* BRB 73, and *Lentinus* sp. BRB 12, as well as evaluate its antibacterial action against various pathogenic bacteria.This research used pathogenic bacteria such as *Staphylococcus aureus*, *Propionibacterium acnes*, *Pseudomonas aeruginosa*, and *Escherichia coli*. The antimicrobial 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. Material and Methods

2.1. Substrate, Microbes, and ChemicalComponents

OPEFB (Oil Palm Empty Fruit Bunch) was collected from an oil palm farm in Cikasungka, West Java, Indonesia., Indonesia. *Trameteshirsuta* D7 (NCBI GenBank, accession No. **KX444204**) was previously isolated from a peat swamp forest region in Riau, Indonesia. *Trameteshirsuta* EDN 082 (NCBI GenBank, accession No.**MT476912**) was isolated from Taman Eden 100, Toba Samosir, North Sumatra, Indonesia. *Leiotrametesmenziesii* BRB 73 (NCBI GenBank, accession No. **MT804553**) and *Lentinus* sp. BRB 12 (NCBI GenBank, accession No. **OR050821**) were isolated from Berbak-Sembilang National Park, Jambi, and South Sumatra, Indonesia. Isolates of *Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus*, and *Propionibacterium acnes*were obtained from the IPBCC (IPB Culture Collection). 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).

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2.2. Fungal ~~Culture~~ =cultivation and Production of Laccase

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Trameteshirsuta D7, *Trameteshirsuta* EDN 082, *Leiotrametesmenziesii* BRB 73, and *Lentinus* sp. BRB 12 was separately cultivated on a PDA medium and culture for 7 d at room temperature (27±3 °C). Laccase was produced via solid-state fermentation using 5 g of

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OPEFB fiber in 100 mL of Erlenmeyer. The OPEFB substrate was then treated with up to 10 mL of MGP medium that consist 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. The mixtures were then sterilized using an autoclave for 15 min and cooled. Six plugs (∅ 5 mm) of the fungal isolate at PDA were put into the sterilized substrate and cultured for 10 d at room temperature (Ningsih*et al*. 2020).

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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 4.5was 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 (YM-10 Amicon, USA) and centrifuged at 4 °C, 10,000 rpm for 10 min. The solution retained on the ultrafiltration membrane is the concentrated crude enzyme and is stored at –20°C.

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2.4. Laccase Characterization

Laccase color, functional groups, enzyme activity, protein content, and enzyme-specific activity were characterized. 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 300–500 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).

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

Where E* is the color value, L* is brightness, a* is redness, and b* is yellowness.

The functional groups of the crude laccase were analyzed using Fourier Transform Infrared (FTIR) (Perkin-Elmer) at 400–4000 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). Protein content was determined using the Bradford technique. 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 wascalculated using Eq. 2.

$$Enzyme\ specific\ activity\ (\frac{U}{mg}) = \frac{Enzyme\ activity\ (\frac{U}{mL})}{Protein\ content\ (\frac{mg}{mL})} \quad (2)$$

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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 ºC) 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 (∅5 mm). 20 µl of Laccase enzyme samples (laccase from *T. hirsuta* D7, *T. hirsuta* EDN 082, *Leiotrametesmenziesii* BRB 73, and *Lentinus* sp. BRB 12) were added separately to each well, and chloramphenicol (CAP) 30 µg/mL used as a positive control ~~were added separately to each well in amounts of 20 µl~~.The plates were placed in an incubator for 24 h at 35±2ºC. By measuring the clear area formed surrounding the hole, the inhibition zone in the medium was estimated (Modarresi-Chahardehi*et al*. 2012).

2.6. Analysis of The Minimum Inhibitory Concentration (MIC)

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The MIC was obtained using the liquid dilution technique.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 (10^8CFU/mL).As a positive control, 500 µL of NB liquid medium was added to 25 µL of bacterial suspension (10^8CFU/mL) in a microplate. Cultures were incubated in an incubator for 24 h at 35±2ºC. 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 30 ºC 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).

3. Result

3.1. Characterization of Laccase Enzyme

T. hirsuta D7, *T. hirsuta* EDN 082, *Leiotrametesmenziesii* BRB 73, and *Lentinus* sp. 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).

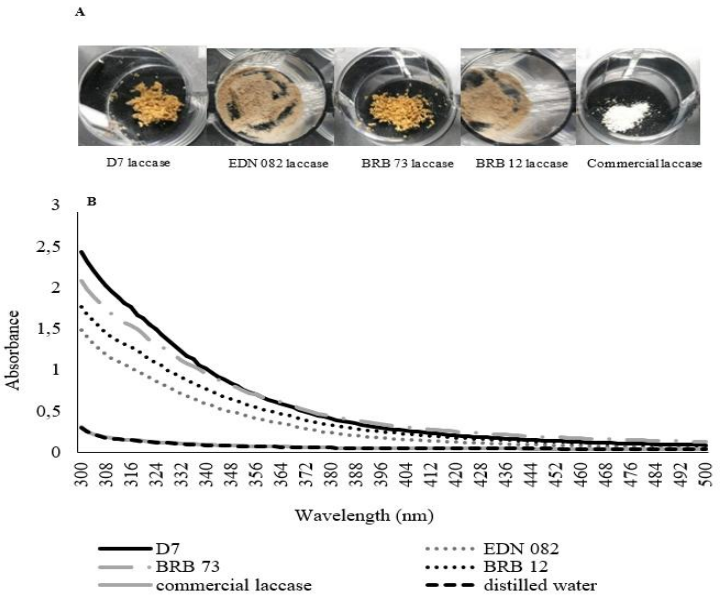

Figure 1. The color (a) and the UV-Vis spectrum (b) of the laccase enzyme

Table 1. Color value of laccase enzyme

Laccase enzyme	L	a	b	E
D7	52.13 ± 0.45	10.13 ± 0.21	19.60 ± 0.35	53.29 ± 0.10
EDN 082	59.27 ± 1.16	10.00 ± 0.17	24.80 ± 0.56	60.31 ± 0.47
BRB 73	54.13 ± 0.45	10.03 ± 0.15	19.60 ± 0.26	55.23 ± 1.16
BRB 12	61.27 ± 1.16	10.07 ± 0.15	24.67 ± 0.67	62.29 ± 0.45
Commercial	91.90 ± 0.10	-0.43 ± 0.06	5.40 ± 0.00	91.93 ± 1.15

The laccase enzyme FTIR spectrum and identified bonds are shown in Figure2 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 groupsand 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) fromamide 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 bending/C-N stretching) from the amide II band.However, some peakswere identical to those of commercial laccases, especially peaks at 1062/1068 cm^{-1} as the characteristic of protein in laccaseand 608/609 $cm^{-1}$from amide V bands.

The peaks of BRB 12 and BRB 73 laccases show a similar pattern. *Leiotrametesmenziesii* BRB 73 and *Lentinus* sp. 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, characteristic of protein in laccases, and the amide VI band, respectively. The three peaks are identical to those of commercial laccases.

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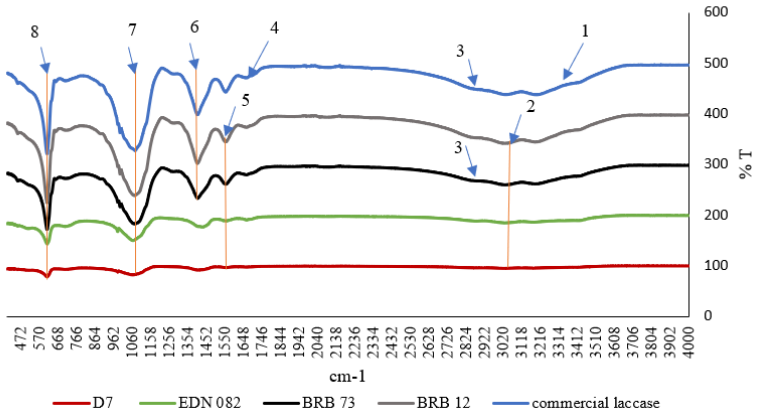

Figure 2. Laccase enzyme FTIR spectrum

Table 2. Functional groups of laccase enzyme FTIR spectrum

No Peak	D7 laccase	EDN 082 laccase	BRB 12 laccase	BRB 73 laccase	Commercial laccase	Functional group assignment (References)	Class compound
	Wave number (cm^{-1})						
1.	-	-	-	-	3298	O-H stretching, N-H stretching	Carbonyl group, Amide A
2.	3061	3179-3033	3193-3028	3197-3030	-	N-H stretching	Amide B
3.	-	-	-	2851	2922	C-H stretching, N-H stretching	CH_2 group
4.	-	-	-	-	1642	C=O stretching vibration, C-N stretching vibration	Amide I with a β-sheet structure
5.	1555	1553	1553	1553	-	N-H bending,C-N stretching vibration	Amide II bands
6.	-	-	1403-1280	1404-1276	1360	N-H bending, C-N stretching	Amide III bands
7.	1062	1068	1069	1094	1015	C-N stretching, C–O–C stretching	Protein in laccase, Aromatic amine
8.	608	609	609	610	572	C=O bending	Amide VI bands

The maximum laccase enzyme activity was obtainedin *Trameteshirsuta* 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.

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 3. The characterization of laccase enzyme produced by *T. hirsuta* D7, *T. hirsuta* EDN 082, *Leiotrametesmenziesii* BRB 73, and *Lentinus* sp. BRB 12

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 *Leiotrametesmenziesii* BRB 73 and *Lentinus* sp. 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/mLsuppressed*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.

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	4.5	0	5.0	8.0	9.0	10.0	7.5
EDN 082	1.0	5.0	0	5.0	8.0	7.0	10.0	7.7
BRB 73	0	1.0	6.5	7.7	9.0	7.0	12.0	9.0
BRB 12	0	0	4.0	5.0	7.0	7.5	10.0	7.7
CAP	12.3	13.0	19.3	11.0	12.3	13.0	19.3	11.0

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*	0.026	7.35	0.0040
		E. coli	0.044	18.36	0.0020
*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
Leiotrametesmenziesii 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 sp. 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
*T. hirsuta*D7	Gram (+)	*S. aureus*	0.044	18.36	0.002

Laccase	**Gram type**	**Test Organism**	**Minimal Bactericidal Concentration (MBC)**

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 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*or and *P. aeruginosa* bacteria.

Table 6. Minimum Bactericidal Concentration (MBC) of laccase enzyme against bacterial strain

		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
Leiotrametesmenziesii 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 sp. BRB 12	Gram (+)	*S. aureus*	0.018	25.40	0.001
		P. acnes	-	-	-
	Gram (-)	*P. aeruginosa*	-	-	-
		E. coli	0.018	25.40	0.001

4. Discussion

Four enzymes produced by four distinct microorganisms were investigated as antibacterial agents. Laccases are produced by *Trameteshirsuta* D7, *Trameteshirsuta* EDN 082, *Leiotrametesmenziesii* BRB 73, and *Lentinus* sp. BRB 12. The laccase enzymes produced by these different kinds of fungi have distinct properties.The laccases produced by all the fungi were brownish-yellow.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 laccasefrom 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 colorabsorption. 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 andJoye 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 ofthe N-H bending and the C-N stretching vibrations gives rise to the amide III bands between 1420 and 1200 cm^{-1} (Bart2007; Samui andSahu 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 regionsbecause 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 at1624 and 1690 cm^{-1} (amide I), 1420-1210 cm^{-1}(amide III), 1165–948 cm^{-1} (characteristic ofprotein in laccase), and at 800–500 cm^{-1} (amide V & VI) (Samui andSahu 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 *Leiotremetesmenziesii* BRB 73 were more comparable to the laccase from *Lentinus* sp. 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.

Trameteshirsuta 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.*Trameteshirsuta* 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 nidulan*s 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 *Pleurotussajor-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 *Pleurotusostreatus* 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, *Leiotrametesmenziesii* BRB 73, and *Lentinus* sp. BRB 12created 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.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

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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.The activity of the laccase enzyme required to suppress Gram-negative bacteria was greater than that required to suppress Gram-positivebacteria. Gram-negative bacteria have a wall and an outer complex membrane while having low levels of peptidoglycan, which contributes to their resilience (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/mLindicated 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).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).

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In conclusion, the four crude laccase enzymes laccase produced by the Indonesian white rot fungus *Trameteshirsuta* D7, *Trameteshirsuta* EDN 082, *Leiotrametesmenziesii* BRB 73, and *Lentinus* sp. BRB 12 inhibited Gram-positive and Gram-negative bacteria. The inhibition effectiveness correlated to the laccase activities applied in the experiment. The concentrated

laccase enzyme's activity increased up to 12-fold and was capable of killing *Propionibacterium acnes* and *Pseudomonas aeruginosa.* Some of these findings contributed to the bioprospection of native Indonesian white rot fungi capable of generating enzymes as antibacterial agents.

Acknowledgment

The project was funded by the National Research and Innovation Agency Republic of Indonesia (BRIN) and used the facility of the Integrated Laboratory for Bioproducts (ILaB) BRIN,through E-Layanan Sains BRIN, Indonesia.

References

AlSheikh, H.M.A., Sultan, I., Kumar, V., Rather, I.A., Al-Sheikh, H., Tasleem Jan, A., Haq, Q.M.R., 2020. Plant-based phytochemicals as possible alternative to antibiotics in combating bacterial drug resistance. *Antibiotics*. 9(8), 480. doi 10.3390/antibiotics9080480.

Anita, S. H., Ardiati, F. C., Oktaviani, M., Sari, F. P., Nurhayat, O. D., Ramadhan, K. P., Yanto, D. H. Y., 2020. Immobilization of laccase from *Trameteshirsuta* EDN 082 in light-expanded clay aggregate for decolorization of Remazol Brilliant Blue R dye. *Bioresour. Technol. Rep*. 12, 100602. https://doi.org/10.1016/j.biteb.2020.100602.

Anita, S.H., Ardiati, F.C., Ramadhan, K.P., Laksana, R.P.B., Sari, F.P., Nurhayat, O.D., Yanto, D.H.Y., 2022. Decolorization of synthetic dyes by tropical fungi isolated from Taman Eden 100, Toba Samosir, North Sumatra, Indonesia. *Hayati J. Biosci*. 29 (4), 417–427. doi:10.4308/hjb.29.4.417-427.

Antecka, A., Blatkiewicz, M., Boruta, T., Górak, A., Ledakowicz, S., 2019. Comparison of downstream processing methods in purification of highly active laccase. *Bioprocess Biosyst. Eng.* 42, 1635–1645. https://doi.org/10.1007/s00449-019-02160-3.

Aruwa, C.E., Amoo, S.O, Koorbanally, N., Kudanga, T., 2022. Laccase-mediated modification of isorhamnetin improves antioxidant and antibacterial activities. *Process Biochem.* 112, 53-61. https://doi.org/10.1016/j.procbio.2021.11.019

Barth, A., 2007. Infrared spectroscopy of proteins. *Biochim. Biophys. Acta*. 1767, 1073–1101. doi:10.1016/j.bbabio.2007.06.004.

Bahanawan, A., Kusumah, A.A., Darmawan, T., Ismadi, Masruchin, N., Sudarmanto, Jayadi, Pramasari, D.A., Triwibowo, D., Kusumaningrum, W.B., Wibowo, E.S., Syamani, F.A.,

Krishanti, N.P.R.A., Lestari, E., Amin, Y., Sufiandi, S., Syahrir, A., Dwianto, W., 2019. Moisture content, color quantification and starch content of oil palm trunk (*Elaeisguineensis* Jacq.). *IOP Conf. Series: Earth Environ. Sci.* 374, 012041. doi:10.1088/1755-1315/374/1/012041.

Baumler, A, Sperandio, V., 2016. Interactions between the microbiota and pathogenic bacteria in the gut. *Nature.* 535, 85–93. doi:10.1038/nature18849.

Becker, D., Giustina, S, V, D., Mozaz, S, R., Schoevaart, R., Baecelo, D., Cazes, M, D., Belleville, M, O., Marcano, J, S., Gunzburg, J, D., Couillerot, O., Volker, J., Oelhmann, J., Wagner, M., 2016. Removal of antibiotics in wastewater by enzymatic treatment with fungal laccase–degradation of compounds does not always eliminate toxicity. *Bioresour. Technol.* 219, 500–509. doi:10.1016/j.biortech.2016.08.004.

Bertrand, B., Martínez-Morales, F., Trejo-Hernández, M. R., 2013. Fungal laccases : induction and production. *Rev. Mex. Ing. Quím.* 12(3), 473–488. http://www.redalyc.org/articulo.oa?id=62029966010.

Besufekad, S.Y., Mekdes, M., Abebech, M., Delesa, D., Tekalign, D., Demitu, K., Birtukan, B., 2017. The antimicrobial activity of leaf extracts of *Myrtus communis*. *J. Microb. Biochem. Technol.* 9(6), 290–292. doi 10.4172/1948-5948.1000380.

Chatterley, A., Laity, P., Holland, C., Weidner, T., Woutersen, S. Giubertoni, G., 2022. Broadband multidimensional spectroscopy identifies the amide II vibrations in silworm films. *Molecules.* 27, 6275. https://doi.org/10.3390/molecules27196275.

Charlotte, J., Hjelholt, P., Crone, F., 2006. Antimicrobial activity of laccases. European Patent, EP 1 030 561 B1, 1–34. https://data.epo.org/publication-server/document?iDocId=2990405&iFormat=2

Dana, M., Khaniki, G. B., Mokhtarieh, A. A., Davarpanah, S. J., 2017. Biotechnological and industrial applications of laccase: A review. *J. Appl. Biotechnol. Rep*. 4(4), 675–679.

Darwish, S.M., Darwish, I.M., 2022. Spectroscopic investigation of TAU protein conformational changes by static magnetic field exposure. *J. Phys. Commun.* 6, 075004. https://doi.org/10.1088/2399-6528/ac7d3a.

Dhevagi, P., Ramya, A., Priyatharshini, S., Geetha Thanuja, K., Ambreetha, S., Nivetha, A., 2021. Industrially important fungal enzymes: productions and applications. In: Yadav, A.N. (Eds.),Recent trends in mycological research. Fungal Biology. Springer, Cham, pp. 263–309.https://doi.org/10.1007/978-3-030-68260-6_11.

Goodell, B., Qian, Y., Jellison, J., 2008. Fungal decay of wood: soft rot, brown rot, white rot. *ACS Symposium Series*, 982, 9—31.doi:10.1021/bk-2008-0982.ch002.

Hidayat, A., Yanto, D.H.Y., 2018. Biodegradation and metabolic pathway of phenanthrene by a new tropical fungus, *Trameteshirsuta* D7. *J. Environ. Chem. Eng*. 6 (2), 2454–2460. doi:10.1016/j.jece.2018.03.051.

Hou, K., Wu, Z.X., Chen, X.Y., Wang, J.Q., Zhang, D., Xiao, C., Zhu, D., Koya, J.B., Wei, L., Li, J., Chen, Z.S., 2022. Microbiota in health and diseases. *Signal Transduct. Target Ther*. 7(1),135. https://doi.org/10.1038/s41392-022-00974-4

Janusz, G., Pawlik, A., Świderska-Burek, U., Polak, J., Sulej, J., Jarosz-Wilkołazka, A., Paszczyński, A., 2020. Laccase properties, physiological functions, and evolution. *Int. J. Mol. Sci.* 21(3), 966. https://doi.org/10.3390/ijms21030966.

Ji, Y., Yang, X., Ji, Z., Zhu, L., Ma, N., Chen, D., Jia, X., Tang, J., Cao, Y., 2020. DFT-calculated IR spectrum amide I, II, and III band contributions of N-Methylacetamide fine components. *ACS Omega.* 5, 8572–8578. https://dx.doi.org/10.1021/acsomega.9b04421.

Khayati, L., H. Warsito, 2016. Keanekaragamanjamurkelas basidiomycetes di kawasanlindung KPHP Sorong Selatan. *ProsidingSymbion.* 213–222. (in Bahasa).

Koyani, R.D., Sharma, R.K., Rajput, K.S., 2014. Biodegradation of synthetic textile dyes by Mn-dependent peroxidase produced by *Phanerochaetechrysosporium. Int. J. Environ. Sci.* 5(3), 652–663. doi:10.6088/ijes.2014050100059.

Mahmud, J., Khan, R.A., 2018. Characterization of natural antimicrobials in food system. *Adv. Microbiol.* 8, 894–916. https://doi.org/10.4236/aim.2018.811060.

Mansur, M., Arias, M.E., Copa-Patino, J.L., Flardh, M., Gonzales, A.E., 2003. The white-rot fungus *Pleurotusostreatus* secretes laccase isozymes with different substrate specificities. *Mycol.*95(6), 1013–1020. https://www.jstor.org/stable/3761909

McLaughlin, J., Watterson, S., Layton, A.M., Bjourson, A.J., Barnard, E., McDowell, A., 2019. *Propionibacterium acnes* and *Acne vulgaris*: New insights from the integration of population genetic, multi-omic, biochemical, and host-microbe studies. *Microorganisms.*7(5),128. doi 10.3390/microorganisms7050128.

Modarresi-Chahardehi, A., Ibrahim, D., Sulaiman, S. F., Mousavi, L., 2012. Screening antimicrobial activity of various extracts of *Urtica dioica*. *Rev. Biol. Trop.* 60(4), 1567–1576. https://doi.org/10.15517/rbt.v60i4.2074.

514 Nandiyanto, A.S.D., Oktiani, R., Ragadhita, R., 2019. How to read and interpret FTIR
515 spectroscope of organic material. Indones. *J. Sci. Technol.* 4(1), 97–118.
516 http://dx.doi.org/10.17509/ijost.v4i1.15806.
517 Neldawati, Ratnawulan, Gusnedi, 2013. Analisis_nilai_absorbansidalampenentuankadar
518 flavonoid untukberbagaijenisdauntanamanobat. *Pillar of Physics.* 2, 76–83. (in Bahasa).
519 Nguyen, L.N., Vu, M.T., Johir, M.A.H., Pathak, N., Zdarta, J., Jesionowski, T., Semblante,
520 G.U., Hai, F.I., Nguyen, H.K.D., Nghiem, L.D., 2020. A Novel approach in crude
521 enzyme laccase production and application in emerging contaminant bioremediation.
522 *Processes*. 8, 648. doi:10.3390/pr8060648.
523 Ningsih, F., Yanto, D. H. Y., Mangunwardoyo, W., Anita, S. H.,Watanabe, T., 2020.
524 Optimization of laccase production from a newly isolated *Trametes* sp. EDN134. *IOP*
525 *Conference Series: Earth Environ.* Sci. 572(1). https://doi.org/10.1088/1755-
526 1315/572/1/012024.
527 Nurhayat, O.D., Ardiati, F.C., Ramadhan, K.P., Anita, S.H., Okano H., Watanabe, T., Yanto,
528 D.H.Y., 2022. Bioprospecting three newly isolated white rot fungi from
529 BerbakSembilang National Park, Indonesia for biodecolorization of anthraquinone and
530 azo dyes. *Biodiversitas.* 23 (2), 613–623. DOI: 10.13057/biodiv/d230201.
531 Rahayu, E., Lahay, N., Jamilah., 2021. Antibacterial inhibition test against the combination
532 extract of moringa leaf (*Moringa oliefera*) and basil leaf (*Ocimumbasilicum*) as a
533 substitute for feed additive. *HAJAS*. 3(2), 85–94. doi:10.20956/hajas.V3i2.20074.
534 Ramadhan, K.P., Anita, S.H., Oktaviani, M., Laksana, R.P.B., Sari, F.P., Nurhayat, O.D.,
535 Yanto, D.H.Y., 2021. Biodecolorization of anthraquinone and azo dyes by newly
536 isolated Indonesia white-rot fungi. *Biosaintifika.* 13(1),16–25.
537 https://journal.unnes.ac.id/nju/index.php/biosaintifika.
538 Robinson, P.K., 2015. Enzymes: principles and biotechnological applications. *Essays*
539 *Biochem*. 59, 1–41. doi 10.1042/BSE0590001.
540 Sadat, A., Joye, I.J., 2020. Peak fitting applied to fourier transform infrared and raman
541 spectroscopic analysis of proteins. *Appl. Sci.* 10, 5918. doi:10.3390/app10175918.
542 Samui, A., Sahu, S. K., 2018. One-pot synthesis of microporous nanoscale metal-organic
543 frameworks conjugated with laccase as a promising biocatalyst. *New J. Chem.* 42(6),
544 4192–4200. https://doi.org/10.1039/c7nj03619a.

Stan, D., Enciu, A-M., Mateescu, A.L., Ion, A.C., Brezeanu, A.C., Stan, D., Tanase, A., 2021. Natural compounds with antimicrobial and antiviral effect and nanocarriers used for their transportation. *Front. Pharmacol.* 12, 723233. doi 10.3389/fphar.2021.723233.

Vivekanandan, K.E., Sivaraj, S., Kumaresan, S., 2014. Characterization and purification of laccase enzyme from *Aspergillus nidulans* CASVK3 from vellar estuary southeast coast of India. *Int. J. Curr. Microbiol. Appl. Sci.* 3(10), 213–227. http://www.ijcmas.com.

Widowati, R., Handayani, S., Al Fikri, A.R., 2021. Phytochemical screening and antibacterial activities of senggani (*Melastomamalabathricum* L.) ethanolic extract leaves. *JIPI,* 26(4), 562–568. doi: 10.18343/jipi.26.4.562.

Yanto, D. H. Y., Guntoro, M. A., Nurhayat, O. D., Anita, S. H., Oktaviani, M., Ramadhan, K. P., Pradipta, M. F., Watanabe, T., 2021. Biodegradation and biodetoxification of batik dye wastewater by laccase from *Trameteshirsuta* EDN 082 immobilised on light expanded clay aggregate. *3 Biotech.* 1–13. https://doi.org/10.1007/s13205-021-02806-8.

Zaccaria, S., Boff, N.A., Bettin, F., Dillon, A.J.P., 2019. Use of micro- and ultrafiltration membranes for concentration of laccase-rich enzymatic extract of *Pleurotussajor-caju* PS-2001 and application in dye decolorization. *Chem. Pap.*https://doi.org/10.1007/s11696-019-00845-3.

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