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Selection of Potential Lignin-Degrading Bacteria and Fungi Isolates from the Gunung Lumut and Sungai Wain in East Kalimantan

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Abstract. The enzymes laccase (Lac), manganese peroxidase (MnP), and lignin peroxidase (LiP) are ligninolytic enzyme groups that have a crucial role in degrading lignin compounds. These complex components are abundant in plant biomass. This research aims to isolate and characterize microorganisms producing the enzymes Lac, MnP, and LiP from leaf litter, rhizosphere soil, and sediment in the Gunung Lumut Protected Forest (GLPF) and Sungai Wain Protected Forest (SWPF), East Kalimantan. The bacterial and fungal isolates obtained were cultured on NA and PDA media enriched with 0.05% guaiacol as a substrate. Initial selection was carried out based on the colony growth rate and the formation of a brownish discoloration zone, which indicates ligninolytic enzyme activity. The superior isolates were then tested for extracellular enzyme activity quantitatively using a UV-Vis spectrophotometer. Among the bacterial isolates, the highest Lac activity is isolated GLS 5.1.2 (173.44 U L^{-1}); the highest MnP activity is isolated GLS 5.3 (177.41 U L^{-1}); and the highest LiP activity is isolated GLS 5.1.2 (1034.05 U L^{-1}). In the group of fungi, isolate SS2 was detected with the highest Lac activity (20.66 U L^{-1}), MnP activity (333.75 U L^{-1}), and LiP activity (2516.13 U L^{-1}). Biodiversity sources in the Gunung Lumut Protected Forest (GLPF) and Sungai Wain Protected Forest (SWPF), East Kalimantan have the potential to produce microbial isolates with unique enzymatic characteristics that have never been reported before. The resulting microbial isolate can be used as a bioactivator in composting organic waste to produce high-quality organic fertilizer.

Keywords: laccase; organic matter; extracellular enzymes

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INTRODUCTION

Safe and sustainable environmental pollution management is highly needed to maintain ecological balance. Environmental pollution is caused, among other things, by organic waste originating from crop residues and agricultural agro-industrial activities (Hajam et al., 2023). Organic waste from farming and forestry activities consists of cellulose (40%-50%), hemicellulose (25-30%), and lignin (15-20%), in addition to proteins and small amounts of ash (Chaurasia, 2019). Lignocellulosic biomass contains complex components to decompose, and leaf litter is a particularly abundant source of lignocellulosic waste. Using microorganisms is an effective way to manage solid organic waste as a source of lignocellulose without harming the environment. There are several obstacles in breaking down lignocellulose, especially the complex structure of lignin, which is protected by cellulose and hemicellulose layers (Thakkar & Bhatt, 2020). Lignin is a component of lignocellulose, an aromatic polymer, not a carbohydrate, making it difficult for microorganisms to degrade (Falade et al., 2017).

Microorganisms from bacterial and fungal groups with lignolytic extracellular enzyme activity play a vital role in breaking down organic materials containing lignin (Suryadi et al., 2022; Illuri et al., 2021). The main extracellular enzymes consist of laccase (EC 1.10.3.2), manganese peroxidase (EC 1.11.1.13), and lignin peroxidase (EC 1.11.1.14). The utilization of bacteria and

fungi with potential extracellular enzyme activity has been widely developed to degrade lignocellulosic biomass sources. Bilal et al., (2023) reported that the bacterial isolate *L. sphaericus* has the ability to produce lignolytic enzymes such as laccase, manganese peroxidase (MnP), and lignin peroxidase (LiP) with good degradation capacity. Laksmi et al., (2023) reported that *Trametes hirsuta* AA-017 is a potential white rot fungus with activity of ligninolytic enzymes, especially laccase enzyme. Fungi are widely known as major producers of lignolytic enzymes; generally, bacteria have a higher growth rate than fungi, and the lignocellulolytic enzymes they produce are resistant to temperature changes (Li et al., 2023; Kumar & Chandra, 2020). Basidiomycete fungi, especially white-rot fungi, are reported to be highly effective in breaking down lignin in solid organic waste (Suryadi et al., 2022).

The effectiveness of microorganisms in breaking down lignin is greatly determined by the ability of each isolate to produce extracellular enzymes. Exploration and isolation of these microorganisms are continuously conducted in various regions to obtain superior isolate candidates. Other factors to consider in bacterial and fungal isolation include non-resistance to antibiotics, non-opportunistic behavior, high stability, and adaptability to the environment. Undisturbed natural conditions have the potential to serve as good habitats for microorganism populations with lignolytic enzyme activity. The Gunung Lumut Protected Forest (GLPF) and Sungai Wain Protected Forest (SWPF) are pristine forest areas that harbor abundant microorganism potential. Forest floors filled with leaf litter provide an ideal habitat for microorganisms with extracellular enzyme activities, especially laccase, MnP, and LiP. The enzyme belongs to the group of lignin-degrading enzymes (LDEs). This potential needs to be explored through the exploration, isolation, and selection of target microorganisms as producers of extracellular enzymes for the development of effective solid organic waste management. This research aims to isolate, characterize, and select microorganisms with lignolytic enzyme activity to obtain bacterial and fungal isolates capable of effectively degrading solid organic waste without harming the environment.

This research enriches our understanding of ligninolytic enzymes (laccase, manganese peroxidase, and lignin peroxidase) produced by microorganisms, especially from Indonesian biological resources that have not been widely explored. This research aims to isolate and characterize microorganisms producing the enzymes Lac, MnP, and LiP from leaf litter, rhizosphere soil, and sediment in the Gunung Lumut Protected Forest (GLPF) and Sungai Wain Protected Forest (SWPF), East Kalimantan. The research results can be a reference for further studies regarding working mechanisms, production optimization, and applications of these enzymes. The resulting microbial isolate can be used as a bioactivator in composting organic waste, which helps farmers produce high-quality organic fertilizer. This reduces dependence on synthetic chemical fertilizers, which has a positive impact on the environment and public health.

METHODS

Media preparation

The materials used include potato dextrose agar (PDA), potato dextrose broth (PDB), nutrient agar (NA), nutrient broth (NB), and veratryl alcohol, MnSO4, H2O2, Chloramphenicol, and guaiacol from Sigma Aldrich. Fungi isolates with laccase enzyme capability are grown on a PDA medium enriched with 0.05% guaiacol (PDA+) refers to the method by Ranimol et al., 2018. Bacterial isolates with laccase enzyme capability are grown on NA medium (5 g L^{-1} Peptone, 5 g L^{-1} Sodium Chloride, 1.5 g L^{-1} Beef extract, 1.5 g L^{-1} Yeast extract, and 22 g L^{-1} agar) enriched with 0.5 ml L^{-1} guaiacol.

The screening of microorganisms producing laccase enzymes

In this study, microorganisms producing lignolytic enzymes, such as laccase, were obtained from exploration in the Gunung Lumut Protected Forest (GLPF) and Sungai Wain Protected Forest (SWPF). Isolation was conducted on decaying leaf litter samples, rhizosphere soil, and sediment in the GLPF and SWPF regions in East Kalimantan. The isolates obtained were confirmed to have lignin-degrading enzyme-producing capabilities, especially laccase, with 12 bacterial isolates and 22 fungal isolates. The initial screening process for bacterial isolates involved growth in NA medium supplemented with 0.05% guaiacol substrate. All fungal isolates were grown on a PDA medium enriched with 0.05% guaiacol substrate and 0.01% (w/v) Chloramphenicol to prevent bacterial growth. Bacterial and fungal groups that showed indications of lignin-degrading enzyme capabilities were then selected

to obtain three superior isolates, each of which was a bioactivator for solid organic waste fermentation. The selection process for microorganisms with the best lignin-degrading enzyme activity was based on colony growth rate and the formation of reddish-brown precipitates (Abd El Monssef et al., 2016).

Qualitative analysis of microorganisms that produce laccase enzymes

All bacterial and fungal isolates that have been screened are then subjected to purification processes and stored on NA (bacterial) and PDA (fungal) media at 4°C. Qualitative testing of growth indices is conducted simultaneously with testing of extracellular laccase enzyme activity, which is indicated by the appearance of a reddish-brown color around the colonies. Oxidation reactions on aromatic compounds from the substrate cause the color change around the colonies. One bacterial isolate is grown on NAS media enriched with 0.05% guaiacol. Bacterial cultures are incubated at 28-30°C for 8 days. The diameter of colony growth and decolorization zone is measured daily, and each tested bacterial isolate is prepared in 3 replicates. Fungal isolates are grown on PDA media supplemented with 0.05% guaiacol substrate (Ranimol et al., 2018). On the 7th day, a small circle measuring 0.8 cm in diameter is taken from the PDA media culture and then grown on PDA media enriched with 0.05% guaiacol as the substrate for evaluating its activity. Fungal cultures are incubated at 32°C for 12 days. Qualitative testing of growth indices and diameter of decolorization zone around fungal isolates are measured daily and prepared in 3 replicates for each isolate.

Quantitative analysis of extracellular enzyme activity

The tested group of extracellular enzymes consists of laccase (Lac), manganese peroxidase (MnP), and lignin peroxidase (LiP). Cultures of bacterial and fungal inoculants with potential as producers of extracellular enzymes (Lac, MnP, and LiP) are grown in 250 ml Erlenmeyer flasks containing 100 ml of respective liquid media. Bacterial isolates are grown in sodium broth (NB) medium, while fungal isolates are grown in potato dextrose broth (PDB) medium. Each isolate is incubated in a shaker incubator at 120 rpm and 28°C. Bacterial cultures are observed until day 8, while fungal cultures are observed until day 12. Laccase, MnP, and LiP enzymes are periodically observed every 24 hours (Ranimol et al. 2018).

Laccase

The determination of laccase enzyme activity (EC 1.10.3.2; Lac) refers to the method by (Chauhan, 2019), using guaiacol as the substrate. The reaction mixture consists of 1 ml of acetate buffer solution, 0.5 ml of guaiacol, and 0.5 ml of crude enzyme extract (supernatant from fungi and bacteria). A blank solution containing 0.5 ml of distilled water (replacing the enzyme) is also prepared. The mixtures are then incubated at 30°C for 15 minutes for fungi and 1.5 minutes for bacteria, followed by a UV-vis spectrophotometer reading the absorbance values at a wavelength (λ) of 420 nm by the formula $E.A = (\Delta A \times Vt \times Df \times 10^6)/(t \times \varepsilon \times d \times vs)$ where E.A = Enzyme Activity (U/ml), ΔA = Absorbance at 420 nm, Df = dilution factor, Vt = Total volume of reaction mixture (ml), v = enzyme volume (ml), t = Incubation time (min) and ε = Extinction Coefficient (M−1 cm−1). d = optical path (cm), vs = sample volume (mL). Laccase activity is calculated using a molar absorptivity coefficient of 12.1 x 10^3 L mol^{-1} cm^{-1} (Chauhan, 2019) .

Manganese peroxidase

The activity of manganese peroxidase (EC 1.11.1.13; MnP) is tested based on the method (Chauhan, 2019), modified using manganese sulfate (MnSO4) as the substrate. Enzyme testing is conducted in a reaction mixture containing 300 µl of pH-adjusted water (pH 4.5), 300 µl of guaiacol (4 mM), 600 µl of MnSO4 (1 mM), 300 µl of crude enzyme extract, and 1200 µl of deionized water. The solution is incubated at 30°C for 2 minutes and then reacted with the addition of 300 µl of hydrogen peroxide (H2O2, 1 mM). The absorbance of the solution is read at λ 465 nm using a UV-Vis Spectrophotometer 1800.

Lignin peroxidase

The activity of lignin peroxidase (EC 1.11.1.14; LiP) is tested based on the method (Chauhan, 2019), using veratryl alcohol as the substrate. Enzyme testing is conducted in a reaction mixture containing 600 µl of citrate buffer solution (0.3 M, pH 4.5), 300 µl of veratryl alcohol (8 mM), 1890 µl of deionized water, and 60 µl of crude enzyme extract. The solution is incubated at 30°C for 2 minutes and then reacted with the addition of 150 µl of hydrogen peroxide (H2O2, 5 mM). The absorbance of the solution is read at λ 440 nm. One unit of laccase, MnP, and LiP activity is defined as the amount of enzyme that transforms 1 µmol of substrate per minute.

Molecular identification of bacterial and fungal isolates

Bacteria

Genomic DNA extraction was performed using Quick-DNA Fungal Bacterial Isolation Kits (Zymo). The 16S rRNA gene marker from selected isolates was amplified using colony PCR protocols. DNA amplification reactions with PCR were carried out with a final reaction volume of 25 µl, with detailed composition as follows: 12.5 µl DreamTaq Green PCR Master Mix (2X), 11.5 µl nuclease-free water, and 0.5 µl of each primer pair. Amplification was performed using forward primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse primer 1492R (5'-GGTTACCTT GTTACGACTT-3'). PCR conditions for the initial denaturation stage were set at 95°C for 1 minute, the primer annealing stage at 95°C for 30 seconds, the elongation stage at 60°C for 30 seconds for 30 cycles, and the final extension stage at 72°C for 30 seconds followed by a final extension at 72°C for 2 minutes. The quantity and quality of PCR products were checked using agarose gel electrophoresis (1% w/v) and stored at -20°C before being sent for Sanger sequencing analysis (Ruhimat et al., 2022).

Fungi

The DNA extraction process for fungi was carried out using the ZYMO RESEARCH Quick-DNA Fungal/Bacterial Kit according to the manufacturer's instructions. DNA amplification of fungi was performed using the TaKara PCR Thermal Cycler. PCR technique was utilized to amplify the target DNA of fungi, specifically the Inter Transcribed Spacer region, which is often used as a marker for fungal identification. Genomic DNA extraction of fungi was amplified using universal primers ITS1 and ITS4. Primer sequences were ITS1 (5’-TCT GTA GGT GAA CCT GCG G-3’) and ITS4 (5’-TCC TCC GCT TAT TGA TAT GC-3’) (White et al. 1990). The amplified DNA was then sent to a sequencing service company to analyze the DNA base sequence of the target isolates.

The sequencing results of the 16S rRNA gene (bacteria) and ITS (fungi) were aligned with the National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST) to determine the closest relatives of each isolate. The sequence alignment process was then conducted using the CLUSTAL application. The Neighbor-Joining phylogenetic tree was constructed using the MEGA VII application with a bootstrap value of 100.

Data Analysis

The data obtained from the calculation and observation of bacteria and fungal populations capable of producing lignin-degrading enzymes are presented in the form of tables and figures. The data were statistically analyzed using one-way ANOVA and continued with the DMRT (Duncan Multiple Range Test) test at a 5% significance level using SPSS 25 if there was a significant difference.

RESULT AND DISCUSSION

Screening microorganisms producing lignin-degrading enzymes

Bacterial and fungal isolates potentially possessing lignin-degrading capabilities were obtained from several ecosystems, including the Sungai Wain Protected Forest (SWPF) and the Gunung Lumut Protected Forest (GLPF) in East Kalimantan. Soil, decomposed leaf litter, and sediment samples from aquatic environments such as rivers and lakes served as the primary sources of samples. Lignin-degrading enzyme activities were tested based on the color change reaction to reddish-brown around bacterial and fungal colonies. Guaiacol was used as a substrate to evaluate lignin degradation activity during the screening process. The brown color change around colonies is attributed to the oxidation process of aromatic organic phenolic compounds, specifically guaiacol, into quinone compounds (Wu et al., 2022).

A total of 156 bacterial isolates and 46 fungal isolates underwent screening to identify lignin-degrading microorganisms. Based on the screening results, 12 bacterial and 22 fungal isolates they exhibited lignin-degrading enzyme activity (Table 1). Isolates obtained from screening were subsequently purified to obtain pure cultures that could be used as stock cultures for further testing.

The successful isolation of bacterial and fungal isolates with lignin-degrading potential underscores the rich microbial diversity present in protected forest ecosystems. These microorganisms hold promise for applications in bioremediation, lignocellulosic biomass conversion, and other biotechnological processes aimed at sustainable environmental management.

Table 1. Sample collection for isolation of lignin-degrading microorganisms

No	Sample Code	Site Selection	Sample Collection	Bacteria isolate	Fungi isolate
1	SWnT	SWPF	Soil	-	6
2	SWnS	SWPF	Litter leaf	1	-
3	GLT	GLPF	Soil	2	-
4	GLS	GLPF	Litter leaf	8	5
5	DB	Bugis River - SWPF	Sediment	-	2
6	DW	Wain River - SWPF	Sediment	-	-
7	SM	Mului River - GLPF	Sediment	-	5
8	SS	Serari River - GLPF	Sediment	1	1
9	PR	All site	Poly-R medium	-	3
			Total	12	22

Qualitative analysis of laccase enzyme-producing microorganisms

The ability to biodegrade lignin naturally is possessed by both bacteria and fungi (Janusz et al., 2017). One of the enzymes that plays a major role in lignin degradation is laccase (Lac). The activity level of laccase enzyme in both bacteria and fungi varies widely, but it is commonly known that fungi are significant producers of lignin-degrading enzymes. The laccase enzyme activity from bacterial isolates isolated from the protected riverine forest area of Sungai Wain (SWPF) and Gunung Lumut (GLPF) was tested on NAS media enriched with 0.05% guaiacol. Isolates showing a color change zone around the colony turning reddish-brown indicate that the isolate is capable of producing laccase enzyme (Senthivelan et al., 2019). Qualitative evaluation is done by observing the colony growth rate of bacteria and the rate of color change (decolorization) that occurs. This selection result is carried out to obtain three candidate bacterial isolates that have the potential to produce laccase enzymes, which can ultimately be utilized to treat agro-industrial waste.

The growth rate of bacterial isolates showed significant results for three bacterial isolates, namely GLS 5.1.2, GLS 5.3, and SWnS 7, with colony diameter values of 0.47 cm, 0.50 cm, and 0.57 cm, respectively (Figure 2. A, B, C). The high growth rate of these three isolates is also accompanied by a significant ability to produce laccase enzymes. The color change rate (decolorization) with the highest activity is possessed by GLS 5.1.2, GLS 5.3, and SWnS 7 with values of 7.33 cm, 7.73 cm, and 8 cm, respectively. This data shows that the growth rate of the three isolates is directly proportional to the rate of color change around the colony. The color change values shown in this study are the main indicators in detecting the presence of laccase enzyme activity in each isolate (Fernández-Remacha et al., 2022).

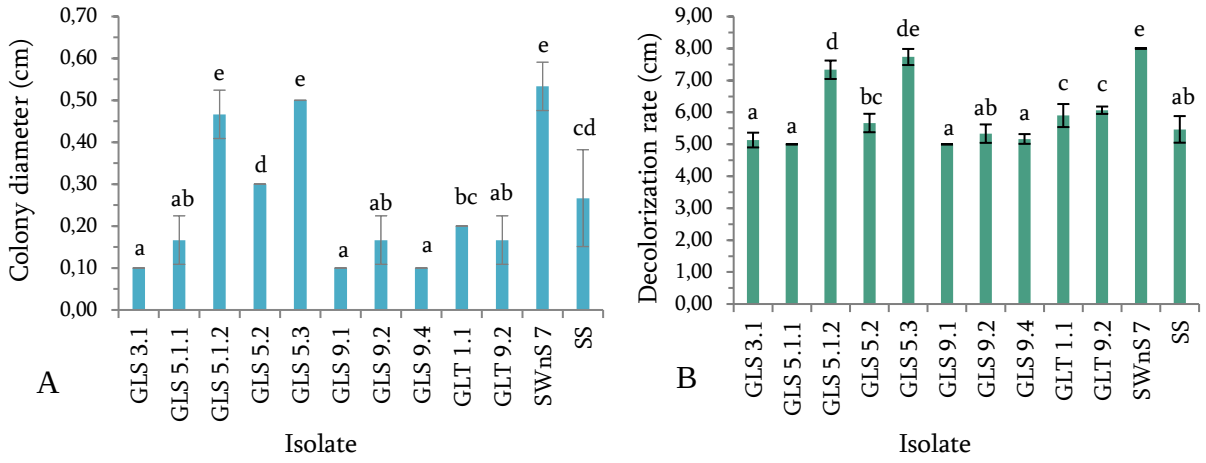


Figure 1. Qualitative selection of bacterial isolates (A) Colony diameter and (B) Rate of color change of 12 laccase enzyme (Lac) producing isolates from the protected riverine forest area of Sungai Wain (SWPF) and Gunung Lumut (GLPF). Note: the same lowercase letter on each bar indicates no statistically significant difference among them ($p < 0.05$).

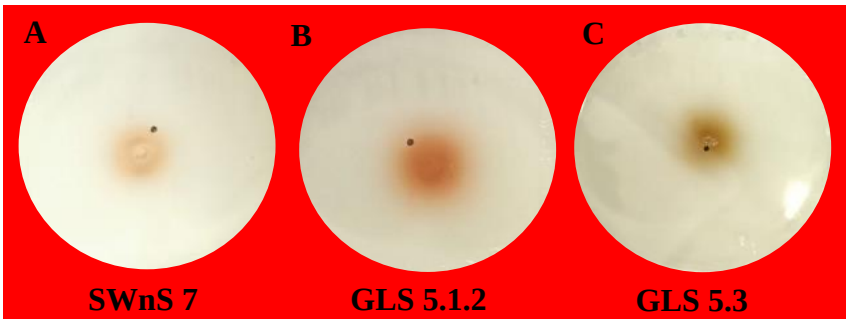


Figure 2. Bacterial isolates with the highest laccase enzyme activity (A) SWnS 7, (B) GLS 5.1.2, (C) GLS 5.3 on solid NAS media enriched with 0.05% guaiacol.

The development of mycelium diameter serves as the basis for evaluating the growth of fungal isolates on agar plates. The mycelial growth of 22 fungal isolates mostly exhibits a relatively similar growth rate (Figure 1.). Based on observations of each fungal colony, it is known that the maximum growth on the 8th day reaches an average of 8 cm. There are 4 isolates that exhibit relatively slow growth, namely SWnT 3, SWnT 6, SWnT, and SM3. Isolates with relatively fast growth have the potential to be selected as candidates if they possess optimum lignin-degrading enzyme activity. Microorganism growth is greatly influenced by medium components such as pH, water activity, and osmotic pressure, as well as external factors such as temperature, oxygen, and pressure. In nature, their growth is heavily influenced by the availability of nutrient sources that can be degraded.

Microorganisms production of laccase (Lac) plays a role in the lignin degradation process. Selection based on laccase enzyme activity aims to obtain the best candidates as lignin-degrading microorganisms. Selection is based on the rate of color change (decolorization) around the colonies forming a reddish-brown color (Figure 2.). The reddish-brown color change serves as an indicator resulting from the use of guaiacol substrate in the medium (Hasan et al., 2023; Senthivelan et al., 2019). The presence of a clear zone or color change in lignin-degrading enzyme activity is influenced by the type of substrate used (Idris et al., 2019). Based on the test results, it is shown that there are 6 isolates with decolorization zone diameters larger than 5 cm, namely SWnT 5 (5.75 cm), GL 3 (5.27 cm), DB 1 (5.22 cm), DB 2 (5.15 cm), SM 5 (6.20 cm), and SS 2 (5.78 cm). The selection of isolates from the top 3 refers to isolates SWnT 5, SM 5, and SS 2, which have higher qualitative lignin degradation activity values compared to other isolates (Figure 2.). These three isolates have the potential for quantitative testing of lignin-degrading enzymes. The extent of color change in the medium indicates high laccase enzyme activity (Fernández-Remacha et al., 2022). Extracellular laccase (Lac) enzymes are highly dependent on solution pH, temperatur and concering storage (Wan et al., 2024; Mehandia et al., 2020).

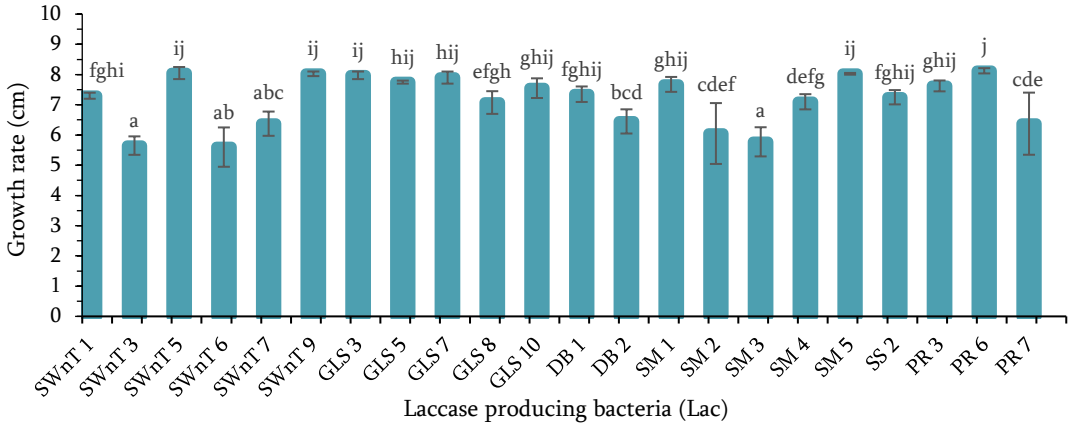


Figure 3. The growth rate of 22 laccase enzyme (Lac) producing isolates from the protected riverine forest area of Sungai Wain Protected Forest (SWPF) and Gunung Lumut Protected Forest (GLPF) after 8 days of incubation. Note: the same lowercase letter on each bar indicates no statistically significant difference among them ($p < 0.05$).

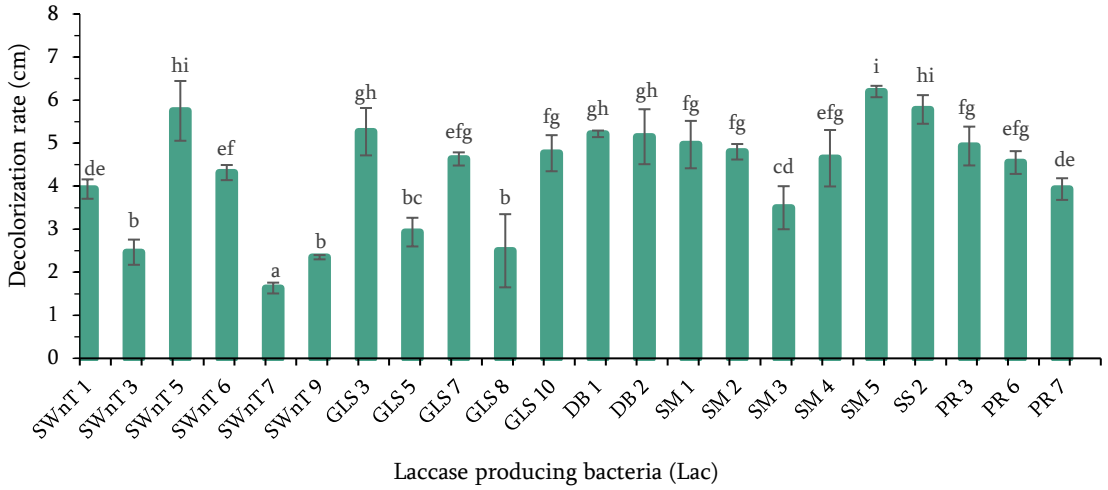


Figure 4. The rate of the color change of 22 laccase enzyme (Lac) producing isolates from the protected riverine forest area of Sungai Wain Protected Forest (SWPF) and Gunung Lumut Protected Forest (GLPF) after 8 days of incubation. Note: the same lowercase letter on each bar indicates no statistically significant difference among them ($p < 0.05$).

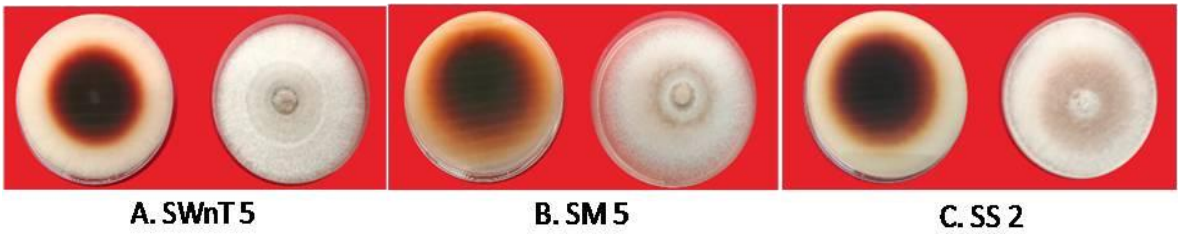


Figure 5. Fungal isolates with the highest laccase enzyme (Lac) activity (A) SWnT 5, (B) SM 5, (C) SS 2 on solid PDA media enriched with 0.05% guaiacol.

Based on observations of the physical structure of the selected fungi, isolates SWnT 5, SM 5, and SS 2 show very significant growth rate and color change (degree of pigmentation) on solid medium (Figure 2). Morphologically, the three selected isolates as lignin degraders from Gunung Lumut (GLPF) and Sungai Wain (SWPF) Protected Forest are seen as white-rot fungi. (Ruhimat et al., 2022) reported that the isolation of fungi with lignin-degrading enzyme activity from Bogor Botanical Gardens produced 3 isolates classified as basidiomycete white-rot fungi, including *Coriolopsis hainanensis, Polyporus thailandensis, and Cerrena aurantiopora*. Previous statements regarding laccase-producing microorganisms reported that white-rot fungi are the main producers of laccase (Lac) that have been extensively studied, and these laccase-producing fungi have been widely used in biotechnological applications (Quintero et al., 2022). Additionally, laccase plays an important role in plant pathogenesis, pigment production, and the degradation of lignocellulose from agricultural waste (Suryadi et al., 2022; Thakkar & Bhatt, 2020).

Quantitative analysis of extracellular enzyme activity.

OD (optical density) values are determined before conducting enzyme activity tests to observe the growth of bacterial cell mass, expressed by OD. A decrease in OD values indicates a decrease in the growth of the bacterial isolate. Optical density (OD) values are measured by measuring the absorbance values of each bacterial isolate culture taken. The results of OD measurements using a spectrophotometer (λ 450 nm) show different absorbance values for each observed isolate sample. The highest absorbance value is obtained from isolate GLS 5.1.2, with an average value of 1.121, followed by isolate SWnS 7 with 71.098, and GLS 5.3 after 4 days of incubation. Isolate GLS 5.3 has the highest OD value of 0.873 CFU Ml-[1] after 3 days of incubation.

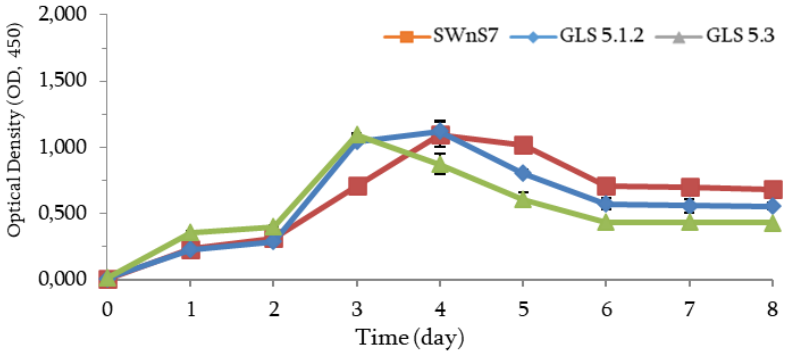


Figure 6. Analysis of optical density (OD) in bacterial isolates producing lignin-degrading enzymes from Sungai Wain Protected Forest (SWPF) and Gunung Lumut Protected Forest (GLPF) during an 8-day incubation period.

The measurement of bacterial biomass weight is based on Figure 7. A shows that isolate SWnS 7 has the highest weight at 95.17 g L^{-1} on day 4 of incubation, followed by isolate GLS 5.1.2 with a weight of 90.17 g L^{-1} on day 4 of incubation. Isolate GLS 5.3 has the highest weight after 5 days of incubation, namely 85.83 g L^{-1}. Based on the results of bacterial biomass weight measurement, it is known that the optimum growth of bacterial isolates occurs during the 3-5 day incubation period. Factors influencing microbial growth can be caused by the type of substrate used, the chemical composition of the media, and environmental factors such as temperature, pH, and oxygen (Gonzalez & Aranda, 2023; Qiu et al., 2022).

Enzymatic evaluation on bacterial isolate cultures was conducted on single cultures of each isolate during a 7-day incubation period in NB (sodium broth) liquid media. The incubation time plays a crucial role in growth and enzyme production (Abd El Monssef et al., 2016). Quantitative testing of laccase, manganese peroxidase, and lignin peroxidase enzymes is important to validate the abilities of each isolate. (Ali et al., 2023; Saskiawan et al., 2021) stated that the role of extracellular enzymes such as laccase, manganese peroxidase, and lignin peroxidase is crucial in degrading lignin compounds. Based on the results of lignin-degrading enzyme (laccase) activity testing, the highest produced by strains SWnS 7, GLS 5.1.2, and GLS 5.3 respectively were 148.32 U L^{-1} in 5 days of incubation, 173.44 U L^{-1} in 5 days of incubation, and 152.36 U L^{-1} in 5 days of incubation (Figure 7. B). The highest level of lignin-degrading enzyme activity (manganese peroxidase) in strains SWnS 7, GLS 5.1.2, and GLS 5.3, respectively, were 118.87 U L^{-1} after three days of incubation, 122.59 U L^{-1} after 3 days of incubation, and 177.41 U L^{-1} after 1 day of incubation (Figure 7. C). The highest level of lignin-degrading enzyme activity in strains SWnS 7, GLS 5.1.2, and GLS 5.3, respectively, were 990.14 U L^{-1} in 5 days of incubation, 1034.05 U L^{-1} in 5 days of incubation, and 956.09 U L^{-1} in 1 day of incubation (Figure 7.D).

The bacterial isolates in this study share the same phylogenetic tree or can be categorized as the same species based on phylogenetic tree identification (Figure 9). However, the similarity in bacterial isolate species as lignin degraders does not indicate the same enzymatic activity. Based on the test results in Figure 7, there are differences in lignin-degrading enzyme activities such as laccase (Lac), manganese peroxidase (MnP), and lignin peroxidase (LiP). This fact is also supported by (Sánchez-Corzo et al., 2021), stating that oxidase enzymes such as MnP and LiP found in fungi from the Ascomycota and Basidiomycota groups are not produced simultaneously or in the same quantities. Highly diverse fungi produce different enzyme combinations, even isolates within the same genus group. For example, isolates with the same genus, such as *Trametes cingulata* TecNM-ITTG L13-19 and *Trametes sanguinea* TecNM-ITTG L14-19, are reported to have different LiP enzyme production values. Although similar in species name, three bacterial isolates originating from SWPF and GLPF areas have the potential to be applied in the degradation of lignocellulosic waste or agricultural waste through solid-state fermentation of organic waste. Based on the enzymatic characteristics of these three isolates, further testing is needed to determine their effectiveness in large-scale degradation. (Al-Zaban et al., 2021) suggest that the development of different combinations of lignin-degrading enzyme-producing isolates is necessary, which can indicate the different abilities of fungi in

degrading media containing lignin or other aromatic compounds as a strategy in the lignin biodegradation process.

Measurement of fungal mycelium biomass weight based on Figure 8. A shows that isolate SWnT 5 has the highest weight of 686.98 g L^{-1} after 12 days of incubation, followed by isolate SM 5 with a weight of 647.55 g L^{-1} after 12 days of incubation. Isolate SS 2 has the highest weight after 10 days of incubation, which is 648.08 g L^{-1}. Based on the results of fungal biomass weight measurement, it is known that optimal growth occurs during the 10-12 day incubation period.

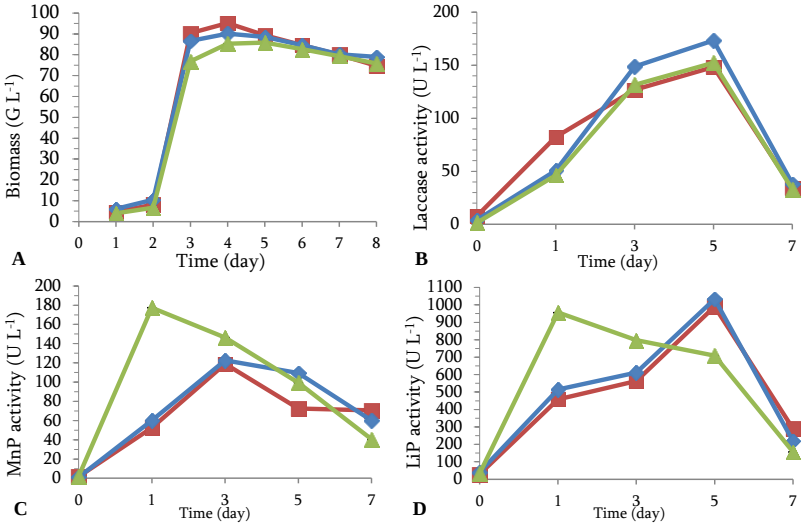


Figure 7. Activities of bacterial isolates producing lignin-degrading enzymes: (A) Mycelium weight, (B) laccase enzyme (Lac), (C) manganese peroxidase enzyme (MnP), and (D) lignin peroxidase enzyme (LiP) from Sungai Wain Protected Forest (SWPF) and Gunung Lumut Protected Forest (GLPF) after 8 day of incubation.

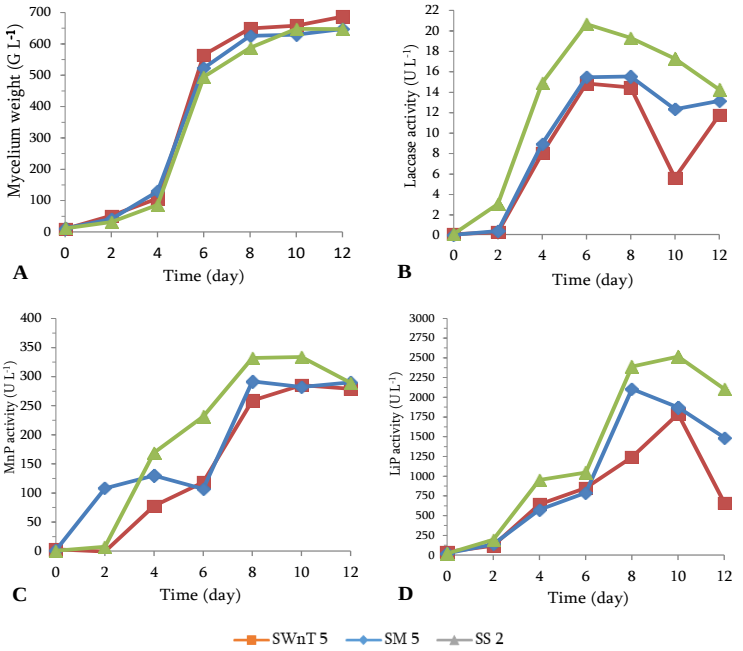


Figure 8. Activity of fungal isolates producing lignin-degrading enzymes: (A) Mycelium weight, (B) laccase enzyme (Lac), (C) manganese peroxidase enzyme (MnP), and (D) lignin peroxidase enzyme (LiP) from Sungai Wain (SWPF) and Gunung Lumut (GLPF) Protected Forest after 8 day of incubation.

The production of lignin-degrading enzymes plays a vital role in fungal growth and assists in degrading lignin contained in solid lignocellulosic waste (Onyancha, 2016). According to Hernández et al., 2017, white-rot fungi naturally are the most efficient microorganisms in producing lignin-degrading enzymes. (Torres-Farradá et al., 2024) have classified white-rot fungi into four groups based on the secreted enzymes: (i) laccase and two peroxidases (MnP, LiP); (ii) laccase and one peroxidase; (iii) only laccase; and (iv) only peroxidase. Selected fungal isolates originating from the Sungai Wain (SWPF) and Gunung Lumut (GLPF) Protected Forest ecosystems, based on enzyme activity testing, fall into group one, meaning they have the ability to produce laccase (Lac), MnP, and LiP enzymes. The highest activity of lignin-degrading enzyme (laccase) produced by strains SWnT 5, SM 5, and SS 2 were 14.85 U L^{-1} in 6 days of incubation, 15.54 U L^{-1} in 8 days of incubation, and 20.66 U L^{-1} in 6 days of incubation, respectively (Figure 8. B). The highest level of lignin-degrading enzyme activity (manganese peroxidase) in strains SWnT 5, SM 5, and SS 2 were 285.54 U L^{-1} after 6 days of incubation, 291.97 U L^{-1} after 8 days of incubation, and 333.75 U L^{-1} after 4 days of incubation, respectively (Figure 8. C). The highest level of lignin-degrading enzyme activity (lignin peroxidase) in strains SWnT 5, SM 5, and SS 2 were 1787.63 U L^{-1} in 10 days of incubation, 2106.63 U L^{-1} in 8 days of incubation, and 2516.13 U L^{-1} in 10 days of incubation, respectively (Figure 8.D). The maximum activity of lignin-degrading enzymes from the GLPF and SWPF areas occurs on days 8-10.

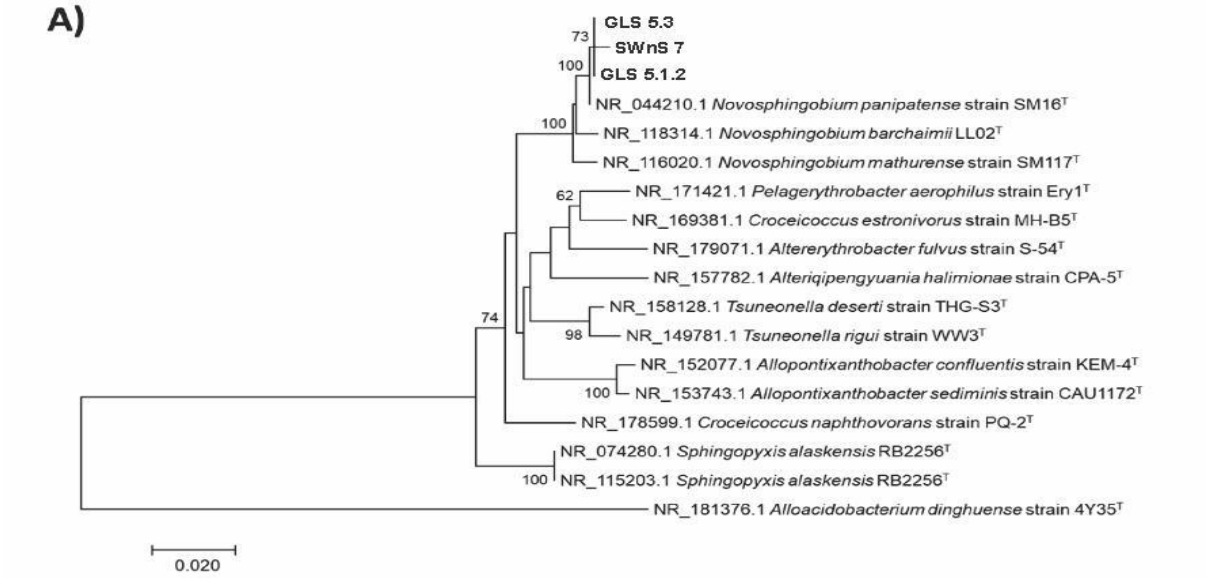
Molecular identification of bacterial and fungal isolates.

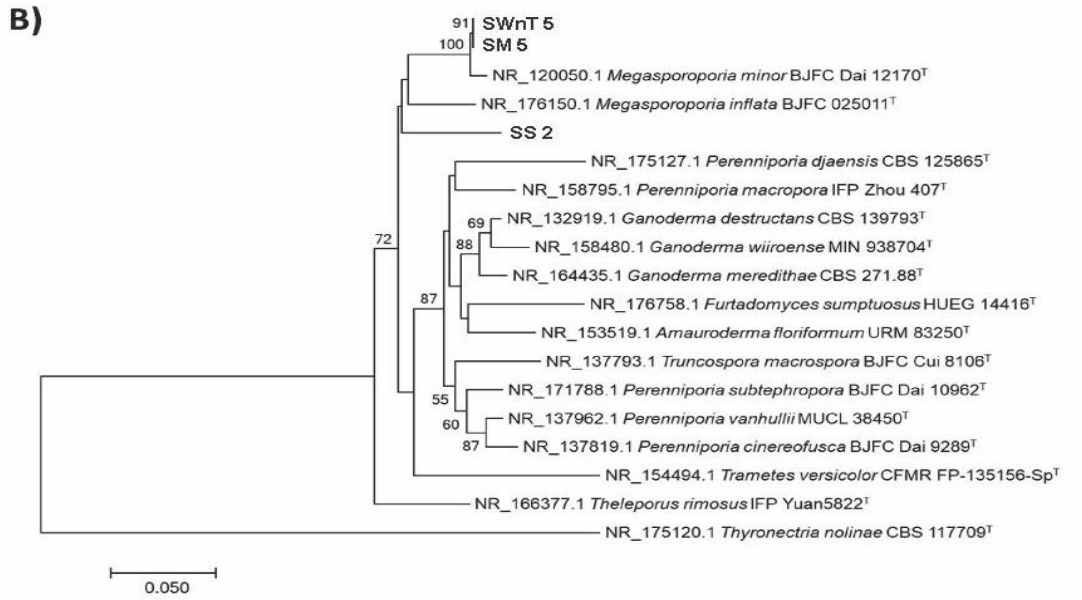
Bacteria

The BLAST results show that the three bacterial isolates, SWnS 7, GLS 5.1.2, and GLS 5.3, have a very close similarity to *Novosphingobium panipatense* SM16T, with similarity values of 99.20%, 99.89%, and 99.90%, respectively. The phylogenetic tree confirms the close relationship of these three bacteria with *Novosphingobium panipatense* SM16T, supported by a bootstrap value of 100 (Figure 9.A). These findings suggest that the three isolates are likely the same species and are classified as *Novosphingobium panipatense*. Further analysis is required to distinguish the metabolic capabilities of the three isolates and determine whether they are different strains of the same species.

Fungi

The fungal isolates SWnT 5 and SM 5 are very similar to *Megasporoporia minor* BJFC Dai 12170T, with a similarity value of 99.10%. Their affiliation with this fungus is further supported by the observation of the phylogenetic tree, with a high bootstrap value of 100 (Figure 9.B). Fungal isolate SS 2 is closely related to *Perenniporia subtephropora* BJFC Dai 10962T (88.43%), *Theleporus rimosus* IFP Yuan5822T (89.68%), and *Megasporoporia inflata* BJFC 025011T (88.67%). However, the phylogenetic tree shows that isolate SS 2 is more closely related to *Megasporoporia inflata* BJFC 025011T, although with a bootstrap value below 50.





Picture 9. The Neighbor Joining phylogenetic tree of bacterial isolates (A) and fungal isolates (B) obtained is shown below. Bootstrap replication was performed using 100 iterations. Only bootstrap values greater than 50 are displayed on the phylogenetic tree.

Isolates of bacteria (Novosphingobium panipatense SM16T) and fungi (egasporoporia minor BJFC Dai 12170T and Megasporoporia inflata BJFC 025011T) are microorganisms that produce lignolytic enzymes (laccase, manganese peroxidase, and lignin peroxidase) which were successfully isolated from areas that have not been widely explored, namely the Gunung Lumut Protected Forest (GLPF) and the Sungai Wain Protected Forest (SWPF) in East Kalimantan. The biodiversity in this area has the potential to produce microbial isolates with unique enzymatic characteristics that have never been studied and reported before. This research can enrich our understanding of ligninolytic enzymes produced by microorganisms, both bacteria and fungi, especially from Indonesian biological resources that have not been widely explored. The research results can be a reference for further studies regarding working mechanisms, production optimization, and applications of these enzymes. The resulting microbial isolates have potential direct applications in solid organic waste management, such as the decomposition of agricultural waste and the production of biofertilizers.

CONCLUSION

Isolates of bacteria and fungi isolated from the Gunung Lumut Protected Forest (GLPF) and Sungai Wain Protected Forest (SWPF) areas have the ability to produce lignin-degrading enzymes. This activity indicates that these isolates naturally have the potential to degrade lignocellulosic-rich agro-industrial waste. Three bacterial isolates with the best activities have been identified, and all of them have similarities with the *Novosphingobium panipatense* strain SM16T. Three fungal isolates with the best activities have been classified as *Megasporoporia minor* BJFC Dai 12170T for isolate codes SWnT 5 and SM 5, while isolate code SS 2 has similarities with *Megasporoporia inflata* BJFC 025011 T. The effectiveness of isolates in lignocellulosic waste degradation needs to be further tested on a larger scale.

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