Malaysian Journal of Chemistry, 2023, Vol. 25(5), 101-106
(Special Issue: *The 4th International Conference on Chemistry and Material Sciences 2022*)

Identification of Indigenous Wood Rot Fungus from East Java Based of ITS Sequence and Their Ligninolytic Activity in Kirk Medium

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Exploration of the ligninolytic indigenous wood rot fungus from tropical areas such as Indonesia has not been widely carried out. The aims of this study were carried out to the species level identification of KLUM1 and KLUM2 from the Kediri Cocoa Plantation and PnUM from the forest of Pujon Malang at East Java Indonesia through phylogenetic analysis based on the Internal Transcribed Sequence (ITS) sequences and identification of their ligninolytic activity in standard Kirk medium. The isolates KLUM1 and KLUM2 were suspected to be novel strains because KLUM1 only had 99% similarity with *Myceliophtora thermophilia* and KLUM2 of 97% with *Thermothelomyces guttulata*, while PnUM was identified as *Phialemonium inflatum* because it had 100 % similarity. All of the three isolates have the ability to produce LiP and MnP which are more dominant than laccase in standard Kirk medium. Thus, the three isolates have the potential to be explored as a source of ligninase enzymes. The exploration of the ligninase ability of these three isolates in various mediums will be interesting for further research in an effort to produce ligninase that is unique and has high activity.

Keywords: Ligninolytic; wood rot fungus; indigenous

Received: September 2022; Accepted: February 2023

Wood rot fungus have the ability to produce lignin-degrading enzymes (ligninase). Ligninase are enzymes that involved in the metabolism of lignin degradation, for self-protection and are involved in the interaction system between fungi [1]. Ligninase can be grouped into phenol oxidase, namely laccase (Lac), and heme peroxidase consisting of lignin peroxidase (LiP), manganese peroxidase (MnP) and versatile oxidase (VP). Laccase (benzenediol: oxygen oxidocreductase E.C. 1.10.3.2) is capable of oxidizing various types of substrates and simultaneously reducing oxygen to water. Lignin peroxidase (E.C. 1.11.1.14) is an extra-cellular heme peroxidase whose activity depends on the presence of H_2O_2, has a high redox potential and is optimum at low pH, can oxidize various reducing substrates including polymer substrates. Manganese peroxidase (EC 1.11.1.13) is an extracellular heme peroxidase which oxidizes Mn^{2+} to Mn^{3+} without the need for H_2O_2, Mn^{3+} reactively forms chelate complexes with short-chain organic acids such as oxalic acid and malic acid. The complex oxidizes the phenolic structure into phenolic radicals which affect the stability of the lignin structure. Versatile oxidase (EC 1.11.1.16) is a newly identified ligninase enzyme capable of oxidizing Mn^{2+} like MnP and has a high redox potential like LiP [2]. Because of their biochemical capabilities, ligninase enzymes are used in various industrial fields including the pulp and paper industry, food, textiles, biosensors, synthesis of organic materials, cosmetics and medicine as well as soil bioremediation [2-4]. Nowadays, *Pycnoporus sanguineus, Pleurotus ostreatus, Lentinula edodes, Lentinus squarrosulus, Psathyrella atroumbonata* and *Phanerochaete chrysosporium* are known as ligninolytic rot fungus. Each species produces the specific type of ligninase which is influenced by the composition of the medium, the strain and growing conditions. *Pycnoporus sanguineus* MTCC-137 produces LiP [5], while *Pleurotus ostreatus* produces MnP [6]. *Lentinula edodes* produces MnP and laccase [7]. *Phanerochaete chrysosporium BKM-F 1767* produced LiP in Kirk's medium [8], produced MnP in Kirk's medium with the addition of Mn (II) [9], produced laccase in Kirk's medium with a modified carbon source in the form of cellulose [10]. The rot fungus that has been widely studied comes from the subtropics areas. There has been no research exploring ligninolytic rot fungus from tropical areas such as Indonesia. In fact, Indonesia is known as an area with mega biodiversity which is thought to also have various types of wood rot fungus that are potential to produce different characteristics ligninase. Indigenous isolates, namely KLUM1

†Paper presented at the 4th International Conference on Chemistry and Material Sciences 2022 (IC2MS2022)

and KLUM2 have been isolated from weathered cocoa husks in the Sepawon Cocoa Plantation, Kediri Regency [11]; and PnUM isolates were isolated from the Pujon Pine Forest, Malang Regency [12]. The three isolates were known to be able to reduce methylene blue in selective media, and based on microscopic observations they were suspected to be related to *Sporotricum aereum*, *Chrysosporium sp* dan *Paecilomyces inflatus* [11], [12]. However, this assumption is not supported by the morphological facts on solid medium because the morphology of the isolates KLUM1, KLUM2 and PnUM on potato dextro agar (PDA) media is different from the morphology of the suspected species so that the species prediction is not acceptable. A more accurate to species identification needs to be done based on the Internal Transcribed Spacer (ITS) DNA sequence. ITS sequences are DNA sequence regions that do not encode functional proteins and are in the ribosomal RNA (rRNA) region. This area is a sustainable area that has a fairly high sequence variation between species and is owned by all fungus so that the ITS area can be used as a genetic marker. The superiority of molecular identification using ITS regional DNA sequences has been proven in several studies, including [13] successfully identifying *Trichoderma sp.* using ITS1 – ITS4 primer pair with 600 bp amplicon results; [14] succeeded in identifying the species *Penicillium sp.* using ITS4 - ITS5 primer pair with 664 bp amplicon results; [15] also succeeded in identifying the kinship of *Phanerochaete chrysosporium* isolates from ITB using ITS4 – ITS5 primers with 700 bp amplicon results. Also, until now there is no information regarding the biochemical potential of the three isolates, especially regarding their ligninase profile in Kirk's medium as the standard medium for ligninase production. Thus, the purpose of this study was to determine the relationship between the indigenous isolates based on ITS sequences with ITS4-ITS5 primers and to determine the biochemical characteristics and potential of these isolates as ligninase producers for further application development.

METHODS

Preparation of the Indigenous of KLUM1, KLUM2 and PnUM Spore Suspension

Cultures of indigenous wood rot fungus of KLUM1, KLUM2, and PnUM isolates were inoculated on PDA slanted agar media. The isolates were incubated at 37 °C for 14 days. Next, the spores were isolated using Tween 80 0.025%, rubbed with an ose needle, left for 5 minutes, the mixture was transferred to a sterile container and homogenized using a vortex for 10 minutes. The mixture was left for 30 minutes, and filtered using a sterile cotton swab in a syringe. The filtrate obtained was a suspension of spore of the indigenous isolates.

Phylogenetic Analysis of the Indigenous of KLUM1, KLUM2 and PnUM Isolates

Indigenous wood rot fungus of KLUM1, KLUM2 and PnUM were grown on slanted PDA for one week at 37 ^{o}C. Genomic DNA was extracted using Quick-DNA Fungal/Bacterial Miniprep KIT. The results were measured by nanodrop to determine the quality and quantity of genomic DNA obtained. DNA amplification used primers ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') with PCR master mix composition and amplification conditions referring to [15]. Purification and sequencing of the amplified results were carried out at Genetica Science Singapore. Alignment using the Bioedit. Phylogenetic analysis was carried out by comparing the ITS4-ITS5 primer sequences from the amplification of local isolates of KLUM1, KLUM2, and PnUM with the ITS4-IT5 primer sequences obtained from the GeneBank data (http://www.ncbi.nlm.nih.gov) using MEGA 5.03 with 1000 bootstrap loops. The number of sequences analyzed were 18 sequences consisting of 3 sample sequences of indigenous isolates KLUM1, KLUM2, and PnUM and 15 reference sequences.

Determine the Ligninolytic Activity of the Indigenous of KLUM1, KLUM2 and PnUM in Standar Kirk Medium.

The spore suspension was grown in standar Kirk Medium to produce ligninase according to the research of [16] with some modifications. The composition of the production media consists of: Basal 1X medium; glucose10 %; trace element solution 1X; thiamine-HCl 0.01%; veratryl alcohol 0.3 g/L; Mn^{2+} 10 mg/L; buffer acetate pH 4.5 0.2 M and ammonium sulfate 20 mM. Three mL of spore culture were put into 20 mL of production medium and incubated at room temperature without shaking. On day 2; 4; 6; and 9 were filtered to obtain the filtrate. The obtained filtrate was measured for protein content and activity of each enzyme, namely lignin peroxidase, manganese peroxidase, and laccase. The repetition was done 3 times.

RESULT AND DISCUSSION

Preparation of the Indigenous of KLUM1, KLUM2 and PnUM Spore Suspension

The KLUM1, KLUM2 and PnUM isolates which had been inoculated on slanted PDA had the same morphological characteristics as those of the pure culture as shown in Figure 1, indicating that the spore colonies were brown in both KLUM1 and KLUM2, and the backs of these two isolates formed a colored crust. dark brown. This is in accordance with [11]. The difference between the two isolates was that in KLUM1 isolates the cells were smoother and the crust formed was not as thick as KLUM2. The results of PnUM inoculated also have a morphology like the

parent culture [12]. Colonies are white, round in shape with grooved edges, wavy margins, and the surface is umbornate (the center appears).

Amplification of ITS Region of the Indigenous of KLUM1, KLUM2 and PnUM Isolates

Genomic DNA obtained using Quick-DNA Fungal/ Bacterial Miniprep KIT had good purity and quantity as a template for amplification using PCR with ITS4 and ITS5 primers (Table 1). The amplicons of KLUM1, KLUM2 and PnUM are ±700 bp (Figure 2). These results are in line with [14] who obtained amplicon with ITS4-ITS5 primer from *Penicillium sp.* by 664 bp and *Phanerochaete chrysosporium* ITB isolates at 700 bp [15].

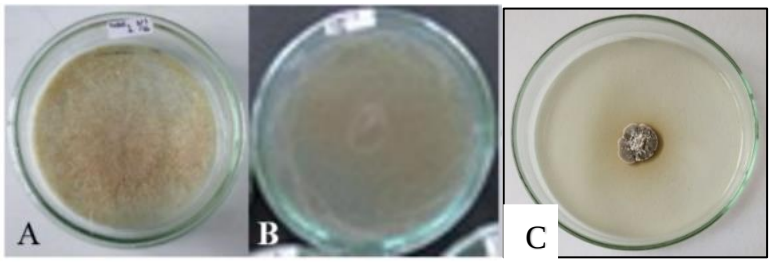

Figure 1. Morphology of inoculated of the indigenous of KLUM1, KLUM2 and PnUM isolates on PDA media.

Tabel 1. Purity and Concentration of KLUM1, KLUM2 and PnUM Genomic DNA.

No.	Sample	Purity of DNA	Concentration of DNA (ng/µl)
1	KLUM1	1.57	203.28
2	KLUM2	1.53	224.32
3	PnUM	1.31	51.51

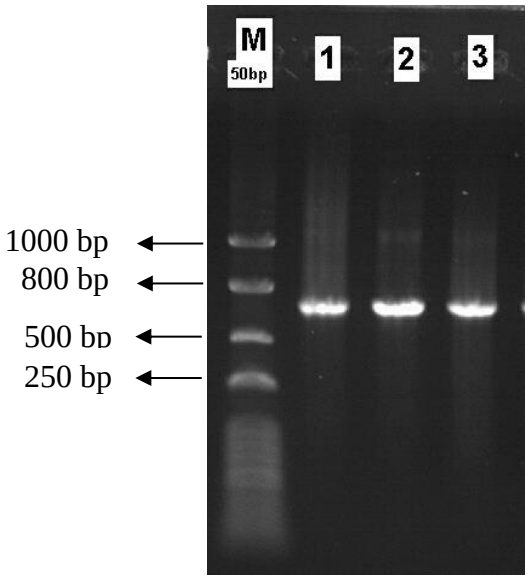

Figure 2. Amplification of ITS region of the indigenous of KLUM1(1), KLUM2(2) and PnUM (3) using ITS4-ITS5 Primer. M: DNA marker.

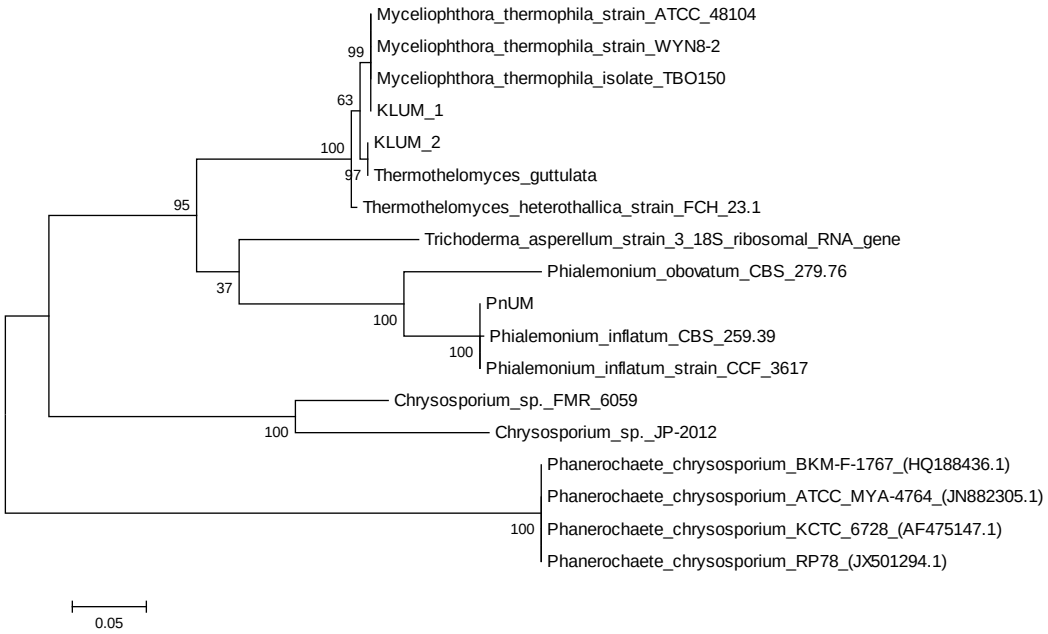

Figure 3. Phylogenetic tree of the indigenous rot fungus compares with reference isolates.

Phylogenetic Analysis of Indigenous Rot Fungus of KLUM1, KLUM2 and PnUM

Reconstruction of phylogenetic trees to determine the species identification of indigenous rot fungus based on ITS regional sequences shows the separation of two large groups (Figure 3). The first group used as an outgroup is the *Phanerochaete*, *Trichoderma* and *Chrysosporium* species. The second group as an ingroup, namely *Myceliophtora*, *Thermothelomyces* and *Phialemonim* according to the results of the BLAST analysis on NCBI using the MOLE-BLAST menu. KLUM1 has a similarity value of 99% with *Myceliopthora thermophilia* species, KLUM2 97% with *Thermothelomyces guttulata* and PnUM 100% with *Phialemonium inflatum*. Shenoy et al., 2007) showed that the isolates KLUM1, KLUM2 and PnUM were the same species as the reference species. *Myceliopthora thermophilia, Thermothelomyces guttulata* and *Phialemonium inflatum* belong to the *Ascomycetes* group, so KLUM1, KLUM2 and PnUM are classified as white rot fungus and have the ability to degrade lignin [17].

Ligninolytic Activity of the Indigenous of KLUM1, KLUM2 and PnUM Isolates in Kirk Medium

Previous study showed that indigenous wood rot fungus known as *Myceliopthora thermophilia* KLUM1, *Thermothelomyces guttulata* KLUM2 and *Phialemonium inflatum* PnUM have the ability to produced ligninase [11, 12], but the information about ligninase profile produced from the three isolates was limited. In this study, the growth curves and ligninase profiles of the three isolates in Kirk's medium were compared with *P. chrysosporium* (the eucalyptus rot fungus that has been widely studied) as a reference. The growth curves showed that the growth profile of *P. chrysosporium* was different from the three isolates, while the three isolates were similar to one another (Figure 4). The increase in protein content of *P. chrysosporium* increased from the second day to the ninth day, while the three isolates experienced a gentle and lower increase than *P. chrysosporium*. This indicates that KLUM1, KLUM2, and PnUM isolates are far apart from *P. chrysosporium* because they show biochemical characters, namely their growth rates in different Kirk medium, but the three isolates are thought to have a close relationship because their growth curves in Kirk medium are almost the same. This result is in line with the phylogenetic analysis that has been carried out (Figure 3).

The indigenous wood rot fungus of KLUM1, KLUM2, and PnUM shown that all of them potentially of producing LiP, MnP, and Laccase as shown in Table 2. The amount of LiP, MnP, and Laccase activity of the three isolates was smaller than that of the control isolate (*P. chrysosporium*). This confirmed the previous assumption that KLUM1, KLUM2, and PnUM isolates were far apart from *P. chrysosporium* because they also showed different biochemical characters (ligninase production). LiP activity produced by KLUM1 was more stable than KLUM2 and PnUM because it appeared on the fourth, sixth, and ninth days. The MnP activity produced by KLUM1 was more stable than KLUM2 and PnUM because it appeared on the fourth and sixth days. Laccase activity of the three isolates only appeared on the sixth day. Based on the results of this study, it is suspected that the indigenous rot fungus studied has the potential to be a source of LiP and MnP enzymes, considering that although the three isolates can produce laccase, their production is unstable and the value is low. The activity of LiP and

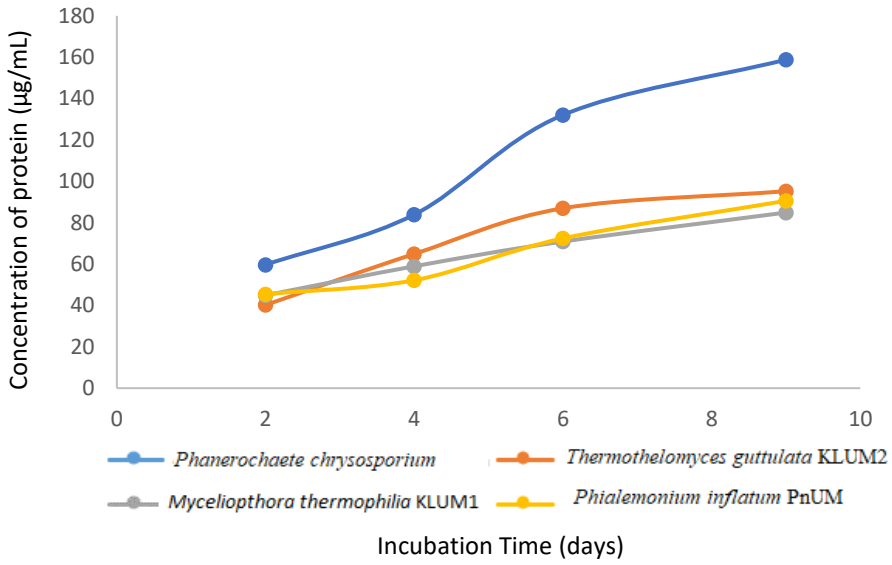

Figure 4. Growth Curves of the indigenous of KLUM1, KLUM2, and PnUM.

Table 2. Activity data tabulation of LiP, MnP, dan laccase from the indigenous of KLUM1, KLUM2, and PnUM isolates.

Isolates	Days	LiP activity (U/mL)	MnP activity (U/mL)	Lacase activity (U/mL)	Results
P. chrysosporium	2	2,15	0	0	*P. chrysosporium* is able to produce LiP > MnP > Laccase
	4	6,45	3,30	0	
	6	8,60	4,96	1,65	
	9	10,75	0	0	
Myceliopthora thermophilia KLUM1	2	0	0	0	*Myceliopthora thermophilia* KLUM1 was able to produce LiP > MnP > Laccase, with a lower activity value than *P. chrysosporium*
	4	2,15	0	0	
	6	4,30	1,65	1,65	
	9	4,30	3,30	0	
Thermothelomyces guttulata KLUM2	2	0	0	0	*Thermothelomyces guttulata* KLUM2 is able to produce LiP > MnP = Laccase, with a lower activity value than *P. chrysosporium*
	4	2,15	0	0	
	6	4,30	1,65	1,65	
	9	0	0	0	
Phialemonium inflatum PnUM	2	0	0	0	*Phialemonium inflatum* PnUM was able to produce LiP > MnP = Laccase, with a lower activity value than *P. chrysosporium*
	4	0	0	0	
	6	2,14	1,65	1,65	
	9	4,30	0	0	

MnP was lower than that of LiP and MnP produced by *P. chrysosporium*, presumably because the medium and conditions of enzyme production carried out in this experiment were optimum for *P. chrysosporium* but not yet optimum for the three isolates.

CONCLUSION

Based on the results of the ITS sequence analysis, indigenous rot fungus KLUM1 and KLUM2 isolated from the Kediri Cocoa Plantation and PnUM from the Pujon Pine Forest were identified as *Myceliophtora thermophilia* KLUM1, *Thermothelomyces guttulate* KLUM2 and *Phialemonium inflatum* PnUM with similarity of the 700 kB of ITS sequences was 99%, 97% and 100%, respectively. All three have the ability to produce LiP and MnP which are more dominant than laccase in standard Kirk medium. For this reason, the three isolates have potential as sources of ligninase enzymes.

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