Conference Paper

Isolation and Screening of Indigenous Fungus Producing Lignin Peroxidase from the Cocoa Plantation in Sepawon Kediri Regency Indonesia

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Abstract

The aim of this research is to obtain indigenous fungus producing lignin peroxidase from the Cocoa Plantation in Sepawon Kediri Regency, Indonesia. Isolation was done using Minimum Salt Medium alkali lignin (MSM-L), purification was performed using monospore technique, lignin peroxidase activity was screened by agar medium containing methylene blue, and microscopic morphology of fungi was studied using slide culture technique. Five isolates of fungus were obtained and three isolates of fungus with different morphology were purified. The screening results showed that only two fungus isolates were indicated as the indigenous fungus producing lignin peroxidase were $KLUM_1$ and $KLUM_2$. $KLUM_1$ was morphologycally predicted as *Sporotricum aereum* and $KLUM_2$ was predicted close to genus *Crysosporium* sp.

Keywords: cocoa; fungus; isolation; lignin peroxidase.

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Received: 9 June 2017
Accepted: 15 July 2017
Published: 11 September 2017

Publishing services provided by Knowledge E

Selection and Peer-review under the responsibility of the NRLS Conference Committee.

1. Introduction

Lignin peroxidase (EC. 1.11.1.14) is oxidoreductase enzyme degrading lignin polymer. It was discovered in *Phanerochaete chrysosporium* by Tien and Kirk in 1983 [1]. Lignin peroxidase catalyzes the oxidative depolymerization of a variety of non-phenolic and phenolic lignin aromatic complex compounds depending on the presence of H_2O_2 [2, 3]. Lignin peroxidase generates cation radicals that can react further through a variety of lignin pathways, including Cα-Cβ cleavage and β-O-4 cleavage on the phenyl propane units leads to the aromatic ring opening [2].

Lignin peroxidase is developed and applied in various industrial fields. Lignin peroxidase replaces the use of chemical compounds for degrading textile dyes and paper waste. It is used as non toxic skin whitening because it has capability to perform oxidative degradation of melanin. Lignin peroxidase is also being developed as a biosensor because of its high redox potential that capable to detect H_2O_2, organic peroxidases polymers, and disease diagnostic compound or antibody label. Lignin peroxidase is

also involved in the production of bioethanol from lignocellulose biomass [3–5]. It is predicted that the future of demand for lignin peroxidase will increase. However, application and utilization of lignin peroxidase are not yet developed properly because of the limited availability of lignin peroxidase. Information about indigeous microbes producing lignin peroxidase and optimization studies evaluating the production of lignin peroxidase is still lacking.

Lignin peroxidase produced by basidiomycetes wood-rot fungi is divided into two types: brown-rot fungi (*e.g. Piptoporus betulinus, Coniophora puteana, Laetiporus sulphures*) and white rot fungi (*e.g Phaenerochaete chrysosporium, Trametes versicolor, Ceriporiopsis subvermispora*). White rot fungi are known to be more effective in degrading lignin than the brown-rot fungi. White rot fungi prefentially degrade lignin leaving the white cellulose and hemicellulose components, while the brown-rot fungi degrade cellulose and hemicellulose leavingbrown lignin in lignocellulosic tissue.

Wood-rot fungi were suspected of numerous in tropical regions that have sufficient moisture in the trunk of rotten wood [6]. One causing of wood decay is the degradation of lignin in the wood. Woods generally contain high amounts of lignin (about 25 %). Lignin in the wood is naturally degraded due to activity of wood rot fungi. Wood-rot fungi which potentially produce lignin peroxidase was estimated live in biomass containing high amounts of lignin. Cocoa weathered has a lignin content of about 38.61 % [7]. This study aimed to obtain indigenous wood-rot fungi from cocoa bark weathered in cocoa Plantation Sepawon Kediri Regency, Indonesia.

2. Methods and materials

The sample sources were cocoa bark that was left until weathered naturally (± two months) in Cocoa Plantation Kediri Regency. Samples were taken using composid technique. Fungus producing lignin was enriched using a medium which lignin provided the sole carbon and energy source (MSM-L) [8]. Enrichment culture were perfomed in 250 mL Erlenmeyer flask by placing 5 g sample in 95 mL MSM-L liquid and the culture were incubated for 14 d at 37 °C in a shaker water bath at 100 rpm (1 rpm = 1/60 Hz). A total of 100 mL of inoculum was transferred into a sterile 0.9 % NaCl and made in a serial dilution of 10^1 to 10^5. A total of 100 mL of each serial dilution was inoculated into agar medium MSM-L using spread method and incubated at 37 °C for 7 d. Then the isolates with different morphology were transferred to the new MSM-L. Purification fungus was done using monospore techniques in PDA and MSM-L agar plate.

The fungus isolates were further screened using methylene blue dye as an indicator: the isolates were streaked on PDA containing 4 % methylene blue and incubated at 37 °C. The agar plates were monitored daily for fungus growth and decolorization of methylene blue dyes. Discoloration of methylene blue or clear zone formed around the

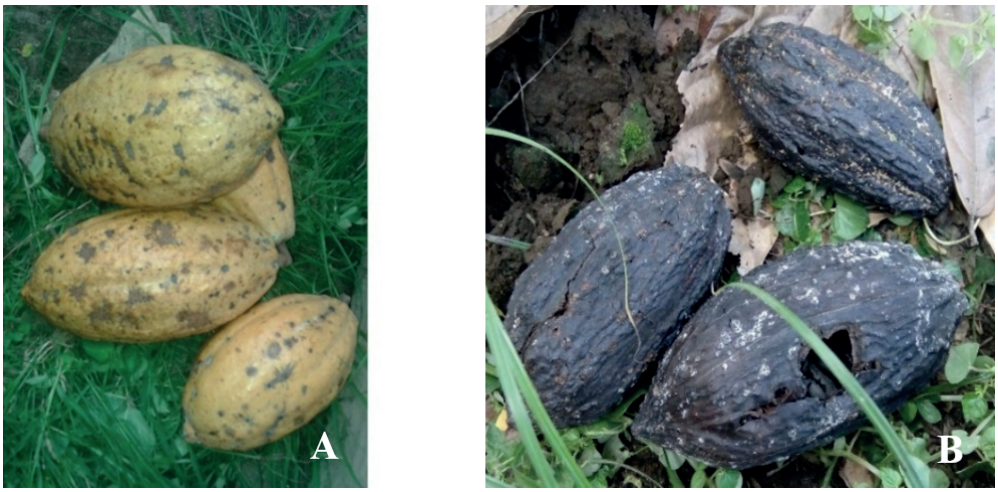


Figure 1: Cocoa Bark: (a) Fresh cocoa bark; (b) Cocoa bark wathered.

fungus growth area is used as an indicator of lignin peroxidase activity. In this method *Phaenerochaete chrysosporium* was used as a positive controls and methylen blue agar medium without the addition of isolate fungus was used as a negative controls. Identity and the characteristics of microscopic fungi were carried by slide culture technique.

3. Results and discussion

The physical changes of cocoa bark weathered is shown in Fig. 1. Cocoa bark changed to dry, brittle, the color changes to brown-black, and on the surface covered with white mycelium fungus.

After the process of enrichment in a liquid medium MSM-L for 14 d, five isolates fungus were obtained (Fig. 2). Each isolate was purified by monospore technique. Isolates 1, 4, and 5 were identified to have similar morphology and labeled as $KLUM_1$, isolate 2 was labeled as $KLUM_2$, and isolate 3 was labeled as $KLUM_3$, monitoring of purification process are shown in Table 1.

Furthermore, the result of screening on the methylene blue agar shows $KLUM_1$ and $KLUM_2$ grow and produce a clear zone while $KLUM_3$ only grow but did not produce a clear zone (Fig. 3). $KLUM_3$were able to grow as a source of sufficient nutrients coming from the media in order to PDA, but did not produce lignin peroxidase and could not degrade methylene blue dye and form a clear zone.

Identification of $KLUM_1$ and $KLUM_2$with slide culture techniques resulted a morphological microscopic image used to predict the genus of fungi by comparing the structure according to literatures, *i.e.*: Pictorical Atlas and Soil Seed Fungi [9], Identification of Fungi Phatogenic [10], and several other references of the fungus structure.

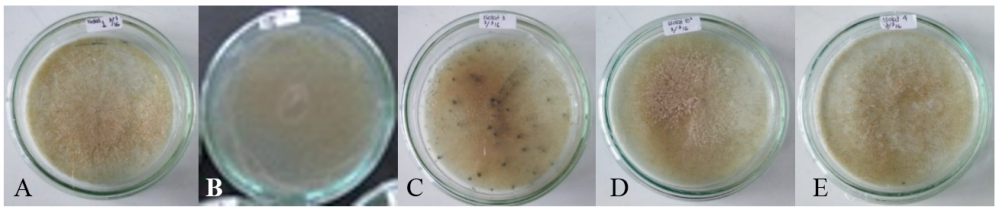


Figure 2: Morphology of isolates 1-5: (a) Isolate 1; (b) Isolate 2; (c) Isolate 3; (d) Isolate 4; (e) Isolate 5.

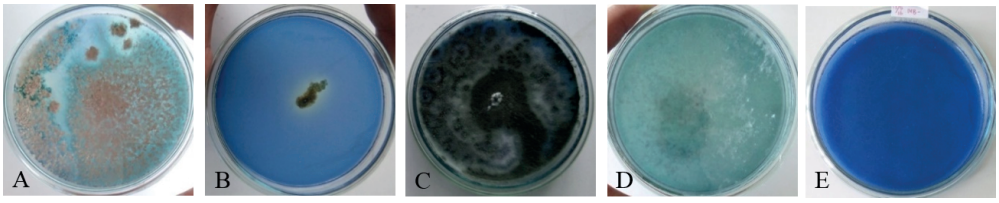


Figure 3: Screens of fungus producing lignin peroxidase: (a) $KLUM_1$; (b) $KLUM_2$; (c) $KLUM_3$; (d) *Phanerochaete crysosporium*; (e) Negative control.

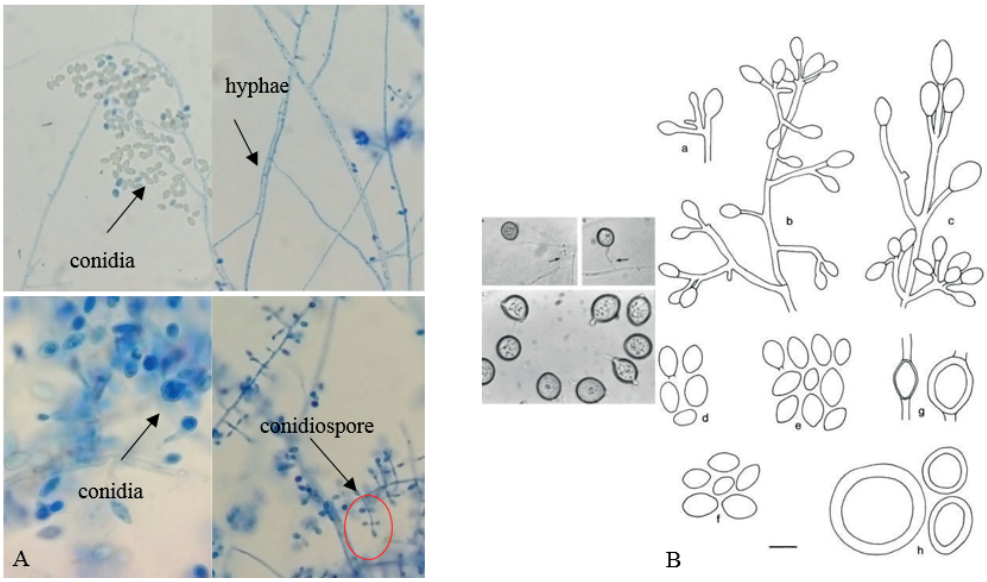


Figure 4: Morphological characteristics: (a) isolate $KLUM_1$; (b) *Sporotricum aerum*.

Morphological analysis of $KLUM_1$ (Fig. 4a) showed that it had septate hyphae, conidiophores short resembles a stalk branching, conidiophores that are not well differentiated from hyphae with clamp connection and conidia globose or ellipsoidal, yellow, thick-walled and readily detached. Based on the references [9–12], $KLUM_1$ had similar morphology with *Sporotrichum aerum* (Fig. 4). The similarities of $KLUM_1$ with *S. aerum* was shown in the shape of conidiophores, conidia that growth branching along the conidiophores, the shape of conidia which have been known for a globose or ellipsodial and yellow [9]. Sporotrichum generally present in wet material plant and rotting or decayed wood [11]. It supports the identification, since the sample source of $KLUM_1$ was cocoa bark weathered.

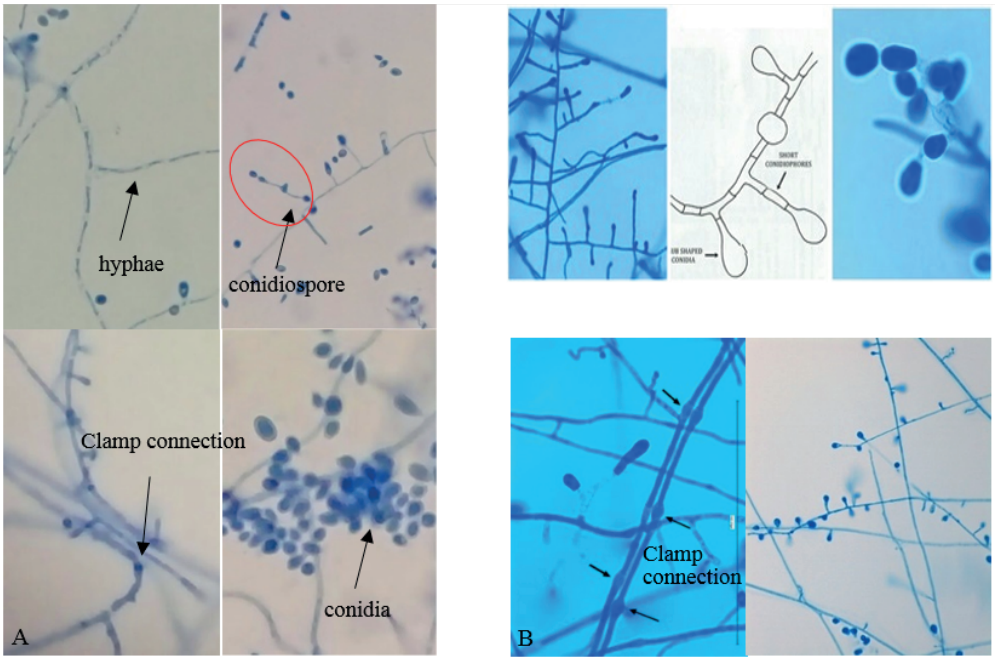


Figure 5: Morphological characteristics: (a) isolate $KLUM_2$; (b) *Chrysosporium* sp.

Based on morphological characteristics analysis of $KLUM_2$ (Fig. 5) showed that it has septate hyphae, the shape of conidiophores elongated, having clamp connection, conidia aleuriosporus, globose or ellipsoidal, thick-walled, readily detached. The study of literature that has been done [9, 10] showed that $KLUM_2$ resemblance to the genus *Crysosporium* sp (Fig. 3). Similarities $KLUM_2$ with *Crysosporium* sp was identified from the shape of conidiophores, conidia aleuriosporus, globose or ellipsodial conidia, and both of them had clamp connection.

Microscopic identification of morphological characteristics of fungus isolate is the fundamental and early step to predict the species. Microscopic identification has possible limitations due to insufficient key structure of fungus which was used for prediction. Repeated observations by varying incubation time is required to determine the initial growth of fungus until the formation of conidiophores, conidia and other complete structures of fungus. Then further identification is required to support the accurate prediction using macroscopic observation by comparing the growth of obtained fungus isolate with the rasemble isolate of fungus reported in the literature using general growth medium agar plate such as PDA under the same conditions. Phylogenetic and further analysis need to be done using specific biochemical testing to ensure the accurate identity of the fungus species.

In this study, the results of analysis showed that $KLUM_1$ morphologycally was predicted as *Sporotricum aereum* and KLUM2 morphologycally was predicted close to genus *Crysosporium* sp. The right identity species of fungus is important before classifying the fungus. Some species of genus *Sporotricum* namely S. *versisporum* was

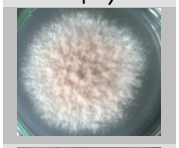
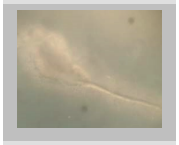
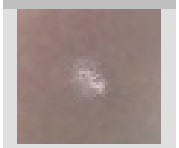
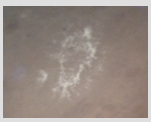
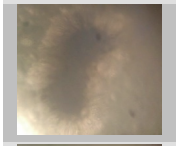
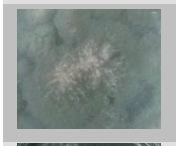
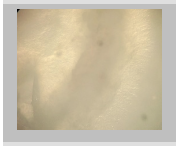
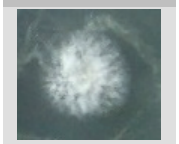
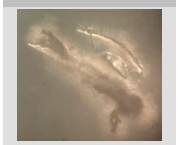
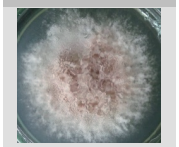
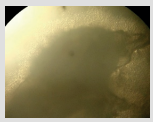
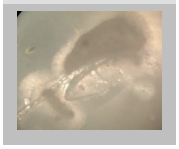
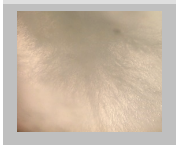
No.	Fungus	Monospore 1		Monospore 2	
		Morphology fungi at PDA/MSM-L agar (incubated 24 h)	Microscopically Image	Morphology fungi at PDA/MSM-L agar (incubated 24 h)	Microscopically Image
1.	Isolate 1 (PDA agar)				
2.	Isolate 2 (MSM-L agar)				
3.	Isolate 3 (PDA agar)				
4.	Isolate 4 (PDA agar)				
5.	Isolate 5 (PDA agar)				

Table 1: Spore morphology isolates fungus.

discovered as a brown-rot fungi, then S. *pruiosum* was discovered as a white rot fungi, while S. *aerum* was not yet discovered clearly [12]. Compared to several other species in the same genus, S. *aerum* is well known as wood-rot fungi. Classification of *Chrysosporium* sp is not yet clear because in this research the prediction is only done in the genus level. In order to get accurately the species identity, identification through phylogenetic level as internal transcribed spacer ITS sequences [13, 14] is very important to do.

4. Conclusion

Two isolate fungi producing lignin peroxidase were obtained from Cocoa Plantation in Sepawon Kediri Regency were $KLUM_1$ and $KLUM_2$. Morphological characteristics of $KLUM_1$ showed that it has septate hyphae, conidiophores short resembles a stalk branching, conidiophores that are not well differentiated from hyphae with clamp connection and conidia globose or ellipsoidal which was yellow, thick-walled and readily detached. $KLUM_1$ was morphologycally predicted as *Sporotricum aereum*. Morphological characteristics of $KLUM_2$ showed that it has septate hyphae, the shape

of conidiophores elongated, having clamp connection, conidia aleuriosporus, globose or ellipsoidal, thick-walled, and readily detached. $KLUM_2$ was morphologycally predicted close to genus *Crysosporium* sp.

Acknowledgements

The author would like to thank Sutoyo for his help and advice during the study of slide culture. We are also thankful to laboratory of microbiology Brawijaya University that gave permission to us to access the laboratory facilities.

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