Isolation, screening, and molecular identification of lignin-degrading fungi isolates from Cibinong Science Centre, Indonesia Institute of Sciences, Cibinong, West Java

P I P A Putri¹, W Mangunwardoyo¹, I M Sudiana², Idris² and I Ramadhanı²

¹Department of Biology, Faculty of Mathematics and Natural Sciences (FMIPA), Universitas Indonesia, Depok 16424, Indonesia
²Microbial Ecology Research Group, Research Center for Biology, Indonesia Institute of Sciences (LIPI), Cibinong 19611, Indonesia

Corresponding author’s email: wibowo.mangun@ui.ac.id

Abstract. Lignin is a recalcitrant compound that has potential as fuels and chemicals on industries. Contamination of recalcitrant compound with the lignin-like structure on the ecosystem is being concerned worldwide. Fungi is the most targeted organisms with lignin-degrading ability, can secrets laccase and peroxidase to facilitate free radical chain reaction on lignin complex structure. Isolation, screening and identification for isolate with ligninolytic ability is the main purpose of this project. Phylum Ascomycota and Basidiomycota be the main target of this research. Isolation technics that being used are surface sterilization with distilled water and NaClO on PDA medium. Pure culture with ability to grow on lignin minimal medium with black liquor as the only carbon sources then are identified by molecular identification. Isolates that can grow on lignin minimal medium are identified as Fusarium verticillioides strain CBS 127178, Lasiodiplodia sp. LAS-2016 strain CBS 125262, Trichoderma harzianum strain 35814DRJ, and Lasiodiplodia theobromae strain CBS 127106. Four isolates obtained with lignin-degrading ability then preserved by cryopreservation technic for use in the next projects.

Keywords: Fungi, isolation, lignin, ligninolytic, molecular identification

1. Introduction
Lignin is the constituent of the plant cell wall along with cellulose and hemicellulose [1]. Plant cell wall is a natural biocomposite, lignin acts as the adhesive to compact the structure. Lignin is a polymer of carbon constructed by monomer, monolignol. Monolignol used in the chemical industries and has a prospect as the sustainable energy resources [2-4]. Extraction of lignin was conducted at 500 °C and high pressure up to 200 bar. Those pre-requirements make the extraction of lignin is difficult to do [5]. A simple method for lignin extraction needs to be explored, for example by using microorganism. Ligninolytic fungi can be the solution for lignin degradation, as they can secrete an extracellular enzyme to facilitate the radical breakdown of recalcitrant bond. Lignin has high durability with cross-link bond of C-C and C-O in aliphatic and aromatic bonds [6].

Lignin makes up 30 % of the stem structure in woody plants, it belongs to the aromatic polymer group constructed by diverse chemical bonds. Chemical linkages within lignin molecules are poorly reactive, preventing any single enzyme to properly degrade it. The non-linear lignin structure is only
capable of being degraded through a radical coupling reaction. Those reactions can be influenced by physical parameters (temperature and dissolved oxygen) and chemical parameters (the concentration of H⁺ ions, monolignol, H₂O₂, oxidative enzymes, and carbon sources) [7]. Radical coupling reaction is an oxidative reaction that involves reactive chemical sites such as hydroxyl group within lignin and saccharides molecules [3].

An example of chemically extracted lignin is black liquor with 90–95 % purity. It is obtained by dissolving lignin-rich materials into acidic solution at high temperature and pressure. Lignin degradation in nature is conducted by ligninolytic microorganisms especially fungi at 20–100 % efficiency. Generally, fungi are the primary lignin degraders. Ligninolytic fungi is already used in various chemical industries such as bioremediation of aromatic compound, bio pulping, and biorefinery for delignifying biomass [8]. Commonly, the lignin production is from pulp and paper industries through spent pulping liquors and chemical discharge of cellulose fibers. Production of lignin-based materials globally exceeds 50 million per annum [3].

Indonesia Culture Collections (InaCC), Indonesia Institute of Science, Cibinong, West Java has many collection of microorganisms: fungi, bacteria, algae, and many other groups. In this project, researchers are going to collect the fungi isolates around the LIPI environment. Isolates that are already proven of having the ligninolytic ability will be identified by the molecular identification method. Screening of ligninolytic ability conducted on lignin minimal medium containing black liquor, ligninolytic fungi can utilize black liquor as primary carbon sources as shown by the capability to grow on lignin minimal medium. Isolates preservation is conducted using cryopreservation methods.

2. Materials and method

2.1. Materials
Research was conducted in June 2018 in the Environmental Microbiology Laboratory, LIPI, Cibinong. Materials being used were: Potato Dextrose Agar (PDA), NaClO [Wako], palm wood dust, salt medium, Plant DNA Extraction Kit [Nucleon Phytopure], Go Taq Green Master Mix, primer ITS4 and ITS5, agarose gel 1 %, TAE buffer, Ethidium Bromide (EtBr), glycerol, trehalose. Software used for molecular identification were: BioEdit v7.0.5, DNA Baser Assembler v4, and webpage ncbi.nlm.nih.gov.

2.2. Black liquor extraction
Black liquor is a dark color liquor contain mostly lignin with a low concentration of hemicellulose. Black liquor was obtained by mixing 250 mL NaOH 1 M with 25 g of palm wood dust, then the solution was incubated for 60 min at 121 °C 2 atm. Hydrolysate was then filtered with Whatman filter paper no. 1, the pH of the medium was adjusted to pH 6.5–7.0 by adding concentrated sulfuric acid (H₂SO₄). Black liquor was stored at temperature 4 °C [5, 9]. Alkali treatment was performed by adding NaOH or other base compounds with high temperature which can solubilize lignin components resulting in black liquor production [8].

2.3. Basal mineral medium
The basal mineral medium contained two types of salt solution: stock I and stock II. Stock I consisted of macromineral (K, Ca, and Mg) (table 1) and stock II contained micromineral (Cu, Fe, Mn, and Zn) (table 2). All solution was sterilized for 15 min at 121 °C 2 atm in the autoclave [10].

2.4. Lignin minimal medium
Lignin minimal medium contained black liquor as the only carbon sources is employed to support the growth of isolates. Medium compositions were: black liquor 1 % (v / v), stock I solution 100 mL/L, stock II solution 1 mL/L, and agar 2 % (w / v). All the components were dissolved in 889 mL aquades to obtain 1 L medium. Medium was then autoclaved for 15 minutes at 121 °C 2 atm. The medium was then distributed on petri dish 90 mm (diameter) with volume 20 mL / petri dish [9, 11].
Table 1. Composition of stock I solution.

| No. | Compound    | Amount (g/L) |
|-----|-------------|--------------|
| 1   | K$_2$HPO$_4$| 45.5         |
| 2   | KH$_2$PO$_4$| 5.3          |
| 3   | CaCl$_2$.2H$_2$O | 5.0      |
| 4   | MgSO$_4$.7H$_2$O | 5.0      |
| 5   | NH$_4$.NO$_3$ | 50.0        |

Table 2. Composition of stock II solution.

| No. | Compound      | Amount (g/L) |
|-----|---------------|--------------|
| 1   | CuSO$_4$.5H$_2$O | 1            |
| 2   | FeSO$_4$.7H$_2$O | 1            |
| 3   | MnSO$_4$.H$_2$O  | 1            |
| 4   | ZnSO$_4$.7H$_2$O | 1            |

2.5. Sampling methods
Sampling was conducted based on the opportunistic method around Cibuntu Lake inside LIPI environment (figure 1). Sample was collected from rotten wood and placed inside a zip lock plastic to prevent cross-contamination.

2.6. Isolation techniques
The medium used for isolation was PDA containing potatoes infusion, glucose, beef extract, and NaCl with pH value 4.5–5.6 [12]. Surface sterilization was conducted by using water and NaClO 2 % for minimalizing the contamination. Fruiting body was sliced to form small squares with dimension 1 x 1 x 1 mm. Four slices were distributed in 4 quadrants on a petri dish. Surface sterilization using NaClO conducted by incubating the slices in NaClO 2 % for 2–3 min while shaking the tube. The fruiting body was then washed using water three times before placing on medium [13]. The plates were incubated at 30–32 °C for 7 days until the colonies appear on the medium. The isolated hyphae were plated onto fresh PDA plates repeatedly to obtain pure cultures [11]. Pure culture was then observed and differentiated based on their colony morphologies.

2.7. Screening for ligninolytic capabilities
Screening was conducted on lignin minimal medium by testing every isolates on lignin minimal medium. Hyphae was obtained from PDA medium after 7–10 days incubation, then picked up by coke borer with diameter 5 mm to minimal medium. Isolates were then incubated for 7 days in incubator at temperature 30–32 °C. Lignin minimal medium containing black liquor acted as the sole carbon and salt medium [11]. Screening on minimal medium only for a pure culture which shows best growth performance on PDA medium. The structure of the hyphae is rigid and tight. Isolates that can grow on lignin minimal medium proved that they can utilize lignin as primary carbon sources [14].

2.8. Molecular identification
Steps for molecular identification including: DNA extraction, Polymerase Chain Reaction (PCR), electrophoresis, and sequencing. DNA was extracted from hyphae of fungi isolates grows on Potato Dextrose Broth (PDB) based on Plant DNA Extraction Kit [Nucleon Phytopure] protocol. DNA
amplification was performed using reverse primer ITS 4 (5’-TCCTCCGCTTATT) and forward ITS 5 (5’-GGAAGTAAAAGTCGTAACAAGG-3’) (figure 2). Reaction mix constitute of 24 μL solution: 12.5 μL Taq polymerase, 1 μL ITS4, 1 μL ITS5, 0.5 μL DMSO, 9 μL nuclease free water, and 1 μL DNA extract. The reaction mix was then placed on the PCR tube and reaction was conducted on the thermal cycler machine. PCR cycle was set for 40s cycle with 5 steps. The early denaturation was performed for 3 minutes on 96 °C, post denaturation for 30 seconds on 96 °C, annealing for 30 seconds on 55 °C, elongation for 1 min 30 sec at 72 °C, renaturation for 10 minutes on 4 °C [13, 15].

Electrophoresis gel compartment was prepared by mixing 1 g agarose powder on 100 mL buffer TAE. Solution mix for electrophoresis contained 2.5 uL DNA mark and 1.5 uL samples. Electrophoresis was run for 20 min at voltage 100 Volt. DNA was visualized by adding Ethidium Bromide (EtBr) until final concentration 0.5 μg/mL for 25–30 min. Gel doc was then being observed on a computer. DNA sequencing was conducted by Macrogen, Korea for knowing the nucleotide sequences.

2.9. DNA sequence analysis
ITS DNA sequences were identified in National Center Biotechnology Information (NCBI) public database with the nucleotide blast (BLASTn) tool. The entries of organisms with high percentage similarity and identity were recorded from the NCBI website [16].

2.10. Sample preservation
Sample preservation was conducted by cryopreservation method with glycerol 10 % and trehalose 5 % and deep freezer (-80 °C). Glycerol are frequently used in cryopreservation of microorganisms and have cell-penetrating capacity at cryogenic temperature (-80 °C) as cryoprotectant [17].

Figure 1. Sampling location in Cibuntu Lake, Indonesia Institute of Science, Cibinong.

Figure 2. Position of primers in the genome of fungi used for amplification of the ITS region.
3. Results and discussion

3.1. Pure culture isolated
Surface sterilization using NaClO aims for sterilizing the fruiting body to minimize the contamination from bacteria, yeast, and fastidious fungi. Husain et al. stated that NaClO has antimicrobial activity, suppress contamination while work aseptically [13]. By using NaClO for washing the sample, result significantly in decreasing of bacterial and yeast contamination. NaClO 2 % can effectively reduce the variation of contaminant, but concentration above it (5.25 %) work better [18]. The purpose of second washing using distilled water is to remove the excess of NaClO that predicted can influence the growth of hyphae. After giving distilled water, the sample needs to be air dried to remove excess of water to minimalize the contamination. Water is important for bacterial growth because it has the high aw value [19].

Epiphytes on fruiting body surface can be cleansed off or be inactivated with surface sterilization, generally using sodium hypochlorite and ethanol to break surface tension. An epiphyte that withstand the surface sterilization and grows in culture can be regarded as an endophyte [20]. In this project, there are 19 samples of fruiting body obtained. Pure culture obtained from purification method are 6 isolates: CSC 01, CSC 06, CSC 09, CSC 14, CSC 18, CSC 19. Those isolates show the growth of single type of hyphae and are the candidate for screening lignin-degrading ability.

3.2. Lignin-degrading fungi isolates
The growth of hyphae on lignin minimal medium shows the morphology color of hyphae, sometimes in white and green colors. Isolates that can grow on lignin minimal medium are 4 isolates: CSC 06, CSC 14, CSC 18, CSC 19. All four isolates grow fast on minimal medium and can fill the petri dish in 7–10 days of incubation. The ligninolytic fungi showed a clear zone of lignin degradation in each plate as shown in figure 3 [11]. The identification of those isolates are: Fusarium verticillioides strain CBS 127178, Lasiodiplodia sp. LAS-2016 strain CBS 125262, Trichoderma harzianum strain 35814DRJ, dan Lasiodiplodia theobromae strain CBS 127106. All of those isolates belong to Ascomycota, sexual reproduction by ascospore inside ascus, also belong to filamentous fungi. The phylum Ascomycota mostly has a limited ability in lignin degradation. It belongs to soft rot fungi. Fungal succession ability on woody plant showed that Ascomycota only takes part in the later stage, while Basidiomycota (which belongs to white rot fungi) dominates wood decomposition on the first stage [4].

Isolates that can grow on lignin minimal medium can degrade lignin, are predicted can secrete the ligninolytic enzymes such as laccase, manganese peroxidase, and lignin peroxidase. The ligninolytic enzyme catalyzes a free radical chain reaction [21]. The presence of Cu²⁺ in basal mineral medium induces the activity of laccase. Laccase contains Cu²⁺ as the active site [8]. Meanwhile, the peroxidase enzyme contains Fe on the heme-prosthetic group [4].

Ascomycota and Basidiomycota have different types of laccase, with a distinct evolutionary histories. Those two types of laccase are differentiated based on their high gene sequence divergences. Clear distinction between extracellular and intracellular laccase enzymes in Trichoderma is identified through comparison in silico analysis. A phylogenetic analysis of Trichoderma shows that two laccase genes is commonly found in those genus, which can be extended to the Ascomycota group that can perpetually loss or gain laccase genes [4].

Lignin-derived aromatic compounds undergo a series of intracellular catabolism reactions which the major pathway is through the β-ketoadipate pathway. β-ketoadipate is the result of β-carboxymuconate reduction through five series enzymatic reactions. The last compound thus formed then undergoes a cleavage to generate succinyl Co-A and acetyl Co-A that eventually will produce ATP [8]. Fungi have diversity of enzyme to degrade carbon polymers. Each enzymes shows a various degradation pattern based on its targeted functional groups within a polymer. Fungal communities influence the decomposition rate, cycling of nutrient and the ecosystem resiliency [22, 23].
Figure 3. Four isolates with lignin-degrading ability: CSC 06; (a) isolated from fruiting body, (b) isolation result on PDA medium, (c) pure culture, (d) grow on lignin minimal medium, CSC 14; (e) isolated from fruiting body, (f) isolation result on PDA medium, (g) pure culture, (h) grow on lignin minimal medium, CSC 18; (i) isolated from fruiting body, (j) isolation result on PDA medium, (k) pure culture, (l) grow on lignin minimal medium, CSC 19; (m) isolated from fruiting body, (n) isolation result on PDA medium, (o) pure culture, (p) grow on lignin minimal medium.

3.3. Species identification
Isolates CSC 06, CSC 14, CSC 18, CSC 19 are continued with molecular identification. The electrophoresis result shows that all the isolates have DNA fragments of 500–750 bp (figure 4). Homology BLAST on ncbi.nlm.nih.gov show identity percentage 98–100 %, high similarities of
Table 3. BLAST results of four isolates DNA sequences from gene bank NCBI.

| No. | Isolates | Species identification          | DNA segment           | Nucleotides | Gaps | Percent of identity |
|-----|----------|--------------------------------|-----------------------|-------------|------|--------------------|
| 1.  | CSC 06   | MH864460.1 Fusarium verticillioides | SSU, ITS1, 5.8s rDNA, ITS2, LSU | 687         | 0    | 100 %              |
| 2.  | CSC 14   | MH863477.1 Lasiodiplodia sp.     | SSU, ITS1, 5.8s rDNA, ITS2, LSU | 543         | 2    | 99 %               |
| 3.  | CSC 18   | MF782823.1 Trichoderma harzianum | 18s rDNA, ITS 1, 5,8s rDNA, ITS2, 28s rDNA | 656         | 4    | 99 %               |
| 4.  | CSC 19   | MH864419.1 Lasiodiplodia theobromae | SSU rDNA, ITS1, 5,8s rDNA, ITS2, LSU | 677         | 3    | 99 %               |

Figure 4. Gel documentation of the four isolates (which are showed by black box).
Code 06b: CSC 06, code 14b: CSC 14, code 18: CSC 18, code 19: CSC 19, M: DNA marker, K: control.

Sequencing result with the DNA sequence of species on the NCBI database. Sequences obtained are on the area of ITS1, 5,8s rDNA, and ITS2 with primer ITS4 and ITS 5 (table 3). Internal transcribed spacer (ITS) is a piece of nonfunctional RNA that shows the highest variation because the fastest rate of evolution. Those codes were chosen as the official barcode for fungi under approval of the Consortium for the Barcode of Life. The first two domains of LSU gene and the entire ITS region have a high prevalence in fungal taxonomy and systematics [24].

4. Conclusion
This little piece of investigation is only a preliminary step to isolate the lignin-degrading fungi. This finding indicates that four isolates obtained, namely Fusarium verticillioides strain CBS 127178, Lasiodiplodia sp. LAS-2016 strain CBS 125262, Trichoderma harzianum strain 35814DRJ, and Lasiodiplodia theobromae strain CBS 127106, have a ligninolytic ability as shown by the growth of
hyphae on lignin minimal medium. Those isolates need further characterization to examine the enzymatic activity for ligninolytic enzyme (laccase, peroxidase, and oxidase).

Acknowledgments
Authors wish to thank sincerely, The Principal of Lembaga Ilmu Pengetahuan Indonesia (LIPI) and InaCC for the support of this research work.

References
[1] Lebo S E, Gargulak J D and MacNally T J 2001 “Lignin”, In: Kirk-Othmer Encyclopedia of Chemical Technology (New York: John Wiley & Sons) 1-2
[2] Ralph J et al. 2001 Phytochemistry 57 993-1003
[3] Feldman D 2016 J. Macromol. Sci. A Pure Appl. Chem. 53 382-7
[4] Janusz G, Pawlik A, Sulej J, Burek U S, Wilkolazka A J and Paszczynski A 2017 FEMS Microbiol. Rev 41 941-62
[5] Barapatre A and Jha H 2017 Biocatal. Biotransfor. 1 269-86
[6] Gaur N, Narasimhulu K and PydiSetty Y 2018 J. Clean. Prod. 198 1602-31
[7] Cesarino I, Araujo P, Junior A P D and Mazzafera P 2012 Braz. J. Bot. 35 303-11
[8] Xu R, Zhang K, Liu P, Han H, Zhao S, Kakade A, Khan A, Du D and Li X 2018 Bioresour. Technol. 269 557-66
[9] Aadil K R, Barapatre A, Meena A S and Jha H 2016 Int. J. Biol. Macromol. 82 39-47
[10] Barapatre A, Meena A S, Mekala S, Das A and Jha H 2016 Int. J. Biol. Macromol. 86 443-53
[11] Sasikumar V, Priya V, Shankar S and Sekar D S 2014 J. Acad. Indus. Res. 3 291-4
[12] Song Z, Liu Q, Guo H, Ju R, Zhao Y, Li and Liu X 2012 Bioresour. Technol. 111 504-6
[13] Husain A, Hassan Z, Faujan N H and Lani M N 2017 Am. Sci. Res. J. Eng. Technol. Sci. 29 182-202
[14] Bugg T.D., Ahmad M, Hardiman E M and Rahmanpour R 2011 Nat. Prod. Rep. 28 1883-96
[15] Schochetman G, Ou C Y and Jones W K 1988 J. Infect. Dis. 158 1154-7
[16] Roslan M A M, Amirudin N A, Abidin Z A Z and Omar S M 2015 J. Teknol. 77 77-81
[17] Prakash O, Nimmonkar N and Shouche Y S 2013 FEMS Microbiol. Lett. 339 1-9
[18] Arnold A E, Maynard Z and Gilbert G S 2001 Mycol. Res. 105 1502-7
[19] Gunasekaran P Laboratory Manual in Microbiology 2005 (New Delhi: New Age International Ltd. Publishers) pp. 20
[20] Alfaro A P and Bayman P 2011 Annu. Rev. Phytopathol. 49 291-315
[21] Nilsson T, Daniel G, Kirk T K and Obst. J R 1989 Holzforschung 43 11-8
[22] Makipaa R, Rajala T, Schigel D, Rinne K T, Pennanen T, Abrego N and Ovaskainen O 2017 ISME J 11 1964-74
[23] Ruel K, Ambert K and Joseleau J 1994 FEMS Microbiol. Rev. 13 241-54
[24] Raja H A, Miller A N, Pearce C J and Oberlies N H 2017 J. Nat. Prod. 80 756-70