Short Communication:
The higher laccase enzyme producer, Cerrena sp. BMd.TA.1, isolated from Gunung Rinjani National Park, West Nusa Tenggara, Indonesia

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Abstract. Faulina SA, Irfani M, Falah S, Hidayat A, Iswanto AH. 2020. Short Communication: The higher laccase enzyme producer, Cerrena sp. BMd. TA.1, isolated from Gunung Rinjani National Park, West Nusa Tenggara, Indonesia. Biodiversitas 21: 3837-3842. Lies in the Wallace line, Gunung Rinjani National Park (GNRP) offers unique biodiversity, fungi included. Fungal enzymes have been unceasingly searched and studied for various applications, particularly for biodegradation. Fungal laccase enzyme showed prospective environmental-friendly approach in treating industrial effluent, remazol brilliant blue R (RBBR) which is used as a synthetic dye. This study aimed to explore the laccase-producing fungi from the GNRP, as well as investigate their ability in decolorizing RBBR. The study discovered that the most prospective fungi isolate, molecularly identified as Cerrena sp. BMd.TA.1, produced a high level of laccase (>2300 U mL\(^{-1}\)) and manganese peroxidase (MnP, 300 U mL\(^{-1}\)). In the application of this isolate, the laccase showed as predominant enzyme in RBBR decolorization process and the RBBR could be decolorized more than 80% at 24 h reaction. It suggested that Cerrena sp BMd.TA.1 isolate is highly potential as laccase enzyme producer and may be considered for further investigations in its applications for biodegradation, especially of dyes effluent.

Keywords: Biodegradation, dye effluent, enzymatic reaction, white-rot fungi

Abbreviations: GNRP: Gunung Rinjani National Park, RBBR: remazol brilliant blue R, WRF: white-rot fungi, MnP: manganese peroxidase, DMP: 2,6-dimethoxyphenol

INTRODUCTION

Indonesia is one of the 17 global megadiverse countries with two of the world’s 25 biodiversity hotspots, and the highest number of protected areas in the term of biodiversity conservation (von Rintelen et al. 2017). As protected areas, national parks serve as field laboratories for biodiversity studies. One of Indonesian national parks is Gunung Rinjani National Park (GNRP), which composed of the tropical rain forests located in West Nusa Tenggara, Indonesia. This area consists of various ecosystem types, ranging from sub-montana to montana forest and savanna (Sadikin et al. 2017). The various ecosystem types resulted in unique biodiversities that have been studied from basic to applied biotechnology (von Rintelen et al. 2017). Darajati et al. (2016) reported that the great potential value of microbial diversity was still underexplored.

One of biotechnology use of microbes, particularly the white rot fungi (WRF), is their ligninolytic enzymes. These enzymes play significant role in mineralizing polycyclic aromatic, polychlorinated, petroleum hydrocarbon, phenolic, and dyestuffs, as well as degrading whole biomass (lignin, cellulose, and hemicellulose) naturally (Wulandari et al. 2014; Sukaryanto et al. 2012; Yanto et al. 2017; Rodriguez-Couto 2017). Other applications in biotechnology are for food, biosensor, biopulping, biobleaching, and biofuel (Septiningrum and Pramuji 2017; Järvinen et al. 2012; Rodríguez-Couto 2017). Among ligninolytic enzymes, laccase and manganese peroxidase (MnP) are widely used in biotechnological application (Falade et al. 2017).

Exploring the substrate-specific laccase and MnP production is very important for industrial application in order to obtain high yield and economic values (Desai and Nityanand 2010). In this study, selection of the prospective WRF isolated from GNRP was screened for their laccase and MnP production, as well as its capability to decolorize remazol brilliant blue R (RBBR) in the fungal culture.
MATERIALS AND METHODS

Sample collections
Eighteen fungi isolate used in this study were isolated and purified from fungal bodies found in Gunung Rinjani National Park (GRNP), West Nusa Tenggara, Indonesia at the elevation of 1000-1500 m a.s.l., that were reached from Senaru Post Main gates (Figure 1). *Trametes hirsuta* D7 was isolated previously and used as control (Yanto et al. 2017). They were grown on potato dextrose agar (PDA), maintained periodically and kept at Indonesian Tropical Forest-Culture Collection (INTROF-CC); Forest Research and Development Centre; Research, Development, and Innovation Agency; Ministry of Environmental and Forestry.

Chemicals
All chemicals used in this study were purchased from Sigma, USA and Himedia, India. Those were 2,2'-azinobis(3-ethylbenzthiazoline)-6-sulphonate (ABTS), agar, glucose, syringaldazine, 2,6-dimethoxyphenol (DMP), gallic acid (GA), and other chemicals at the highest purity available.

Fungal selection and identification
Eighteen fungal isolates with addition *T. hirsuta* D7 isolate as the positive control, were initially evaluated for its ability to decolorize 100 mg L\(^{-1}\) of RBBR in PDA media containing glucose (20 g L\(^{-1}\)), potato (200 g L\(^{-1}\)), and agar (15 g L\(^{-1}\)). The most active isolates in comparison to positive control were chosen and screened based on ligninolytic enzyme production by three qualitative methods (Lonergan et al. 1993; Hidayat and Tachibana 2013). PDA was used as the fungal culture medium, with addition of dimethoxyphenol (DMP), gallic acid, or syringaldazine. The cultures were incubated at 25 °C for 7 d in the first selection, and 4-6 d in the second selection. The MnP and laccase enzyme productions were quantified in the last screening. The fungal isolates with color appearance and mycelium growth ratio more than 100% were chosen and cultured into 20 mL of potato dextrose broth (PDB) in 100 mL Erlenmeyer flasks. After incubation for 7 d at 30 °C, the liquid culture from each sample was subjected for enzymatic assay.

Molecular approach was employed for fungal identification. DNA was obtained from mycelia cultured in PDB for 7 d and extracted using DNA Wizard Kit (Promega, USA) according to the method described by manufacturer. The internal transcribed spacer ITS region (Jellison and Jasalavich 2000) of ribosomal RNA genes was amplified by polymerase chain reaction (PCR) using ITS1 and ITS4 (White et al. 1990). DNA extracts were amplified with Go Tag® Green Master Mix (Promega, USA) according to the manufacturer’s instructions. The purified PCR products were Sanger sequenced using the same PCR primers (First Base Sequencing Service, Singapore). Sequences were aligned and compared by BLAST searches in the National Center for Biotechnology Information (NCBI) GenBank database (http://www.ncbi.nlm.nih.gov/). The phylogenetic tree was constructed with maximum likelihood method using MEGA 7 software.

Figure 1. Map of sampling location in Gunung Rinjani National Park, West Nusa Tenggara, Indonesia
The decolorization of RBBR was evaluated using PDB as culture medium. The amount of RBBR in the culture was 100 mg/L. Dyes were added after 2, 4, and 6 d of Cerrena sp. BMD. TA. 1 cultivation. After dye was added, the mix was incubated for additional 24 h before measurement. Five milliliters of culture mix was sampled and centrifuged at 8,000 g. The decrease in supernatants absorbance at the absorbance maximum (λmax) 595 nm was measured using a UV-visible spectrophotometer and monitored. The decolorization was calculated by following formulation:

% Decolorization = \[1 - \frac{C}{C_0}\] \times 100%

Where, C and C0 refer to initial and final absorbance at 595 nm, respectively. C and C0 refer to absorbance dye before and after decolorization at each sampling time. Control was prepared with fungi culture without dye.

**Enzymes assays**

The enzymatic activities were quantified directly after decolorization analysis (as mention in section above). Activities of MnP and laccase were determined by measuring the increase of optical density at 30°C. MnP activity was assayed using 50 mM malonate buffer and dimethoxyphenol in 20 mM of manganese sulfate (MnSO₄) at 470 nm (Warishishi et al. 1992). Laccase activity was assayed by measuring the increase in absorbance at 525 nm using syringaldazine as a substrate in sodium acetate buffer (Leonowicz and Grzywnowics 1981). Activities were expressed as international units per liter of enzyme, where one unit of activity is defined as the amount of enzyme required to convert 1 µmol of substrate in 1 min.

**Data analysis**

All results were presented as the mean ± the standard deviation and calculated by using Microsoft Excel program.
Figure 3. The effects of various substrates on ratio of decolorization and fungal growth. Mean of ratio is the average of quantitative value of all substrates. Syringaldazine, Gallic acid, 2,6-DMP were measured at 6 d, 4 d, and 5 d, respectively.

Figure 4. Laccase and MnP activity at liquid culture after 7 d.

Figure 5. Phylogenetic relationship of *Cerrena* sp. BMd.TA.1 and related species sequences. The numbers in parentheses are NCBI accession number.
Decolorization of RBBR by fungal culture BMd.TA.1

The capability of Cerrena sp. BMd.TA.1 in decolorizing RBBR was also investigated. The dye solution was added at 2-d intervals pre-incubation and analyzed 24 h after added to test total dye absorbance. Cerrena sp. BMd.TA.1 was able to decolorize RBBR more than 80% after 24 h (Figure 6). When pre-incubation of fungal Cerrena sp. BMd.TA.1 was extended (to 4 and 6 d), the decolorization increased about 10% than shorter pre-incubation (2 d).

Discussion

BMd.TA.1 was identified Cerrena sp. Generally, morphology of genus Cerrena is characterized through dimitic or trimitic hypal system, nonamyloid, hyaline basidiospore, and white rot habitat (Lee and Lim 2010). Cerrena and Trametes were closely related, but the bipolar mating type and clarified generative hyphae of Cerrena could be distinguished as a separate genus (Ryvarden 1984). In previous studies, Cerrena sp. was reported to produce laccase enzyme with activity up to 400 U mL⁻¹ (Sonulashevili et al. 2015; Kachlishvili et al. 2014; Yang et al. 2014; Hidayat and Tachibana 2013). In this study showed that BMd.TA.1 isolate produced the highest laccase enzyme (2300 U mL⁻¹, Figure 4), even when it was cultivated in liquid culture without the addition of enzyme co-factors and inducers. Moreover, this isolate also had highest MnP enzyme activity among isolates tested in this study.

Cerrena sp. BMd.TA.1 was able to decolorize RBBR in liquid culture in this study. The decolorization was increased with the addition of pre-incubation time. Previous result showed that RBBR dye was decolorized by laccase from Cerrena unicolor, Cerrena sp. HYBO7 and fungal culture Cerrena sp. F0607 (Moilanen et al. 2010; Hidayat and Tachibana 2013; Yang et al. 2014). Other studies described that the decolorization of dyes using living fungal should consider bisorption mechanism, which was about 5-50% of total color removal (Mou et al. 1991; Hadibarata et al. 2012a). In our studies, it showed that the isolate could decolorize dyes but it was not significantly tough enough as dye removal, because the color of mycelium of fungal clearly the same as those of the control. Furthermore, RBBR decolorization is used to estimate and select the ligninolytic enzyme activities. Two ligninolytic enzymes were detected during RBBR decolorization by Cerrena sp. BMd.TA.1, those are laccase and MnP. The highest enzyme activity was monitored from laccase and showed in increasing activity when incubation length was extended (Figure 6). These results revealed that the laccase has an important role in breaking down and decolorizing RBBR.

Decolorization rate was obtained as result of the decreased visible absorbance, changing the blue color to colorless. Decolorization also indicated that the chromophoric group was broken down into several RBBR metabolite products (Hadibarata et al. 2012b). More details, laccase was able to oxidize RBBR to intermediate product, sodium 1-amino-9,10-dioxo-9,10-dihy-droanthracene-2-sulfonate (m/z = 341, H) and sodium 2-((3-aminophenyl) sulfonyl)ethyl sulfate (m/z = 303, H) (Sari et al. 2012; Hadibarata et al. 2012b). Even though, the pathway of RBBR transformation by Cerrena sp. BMd.TA.1 was not investigated in this study, the decolorization was done truly by this fungus (Figure 6). Decolorization of RBBR is a simple indication through enzymatic reaction and could be used as an approach to determine the ability of fungi in xenobiotic biodegradation studies (Machado et al. 2005). According to the results in this study, the fungus Cerrena sp BMd.TA.1 isolated from GRNP, Senaru section particularly, promises as an alternative fungal agent for detoxification and decolorization of dyes effluent as well as on the biodegradation application in the broader areas.

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REFERENCES

Darajati W, Pratiwi S, Herwinda E, Radiansyah AD, Nalang VS, Nooryanto B, Rahajoe JS, Ubadillah R, Maryanto I, Kurniawan R, Prasetyo TA, Rahim A, Jefferson J, Hakim F. 2016. Indonesia Biodiversity Strategy and Action Plan 2015-2020. Kementerian Perencanaan Pembangunan Nasional/BAPPENAS, Indonesia [Indonesian].

Desai SS, and Nityanand, C. 2011. Microbial laccases and their applications: a review. Asian J Biotechnol 3 (2): 98-124.

Falade AO, Nwodo UU, Iwierzieb BC, Green E., Mahinya LV, Okoh AI. 2017. Lignin peroxidase functionalities and prospective applications. Microbiol Open 6 (1): e00394.

Hadibarata T, Yusoff ARM, and Kristanti RA. 2012b. Decolorization and metabolism of anthraquinone-type dye by laccase of white-rot fungi Polyporus sp. S133. Water Air Soil Pollut 223(2): 933-941.

Hadibarata T, Yusoff ARM, Arais A, Salmati, Hidayat T, Kristanti RA. 2012a. Decolorization of azo, triphenylmethane, and anthraquinone dyes by laccase of a newly isolated Armillaria sp. F0022. Water Air Soil Pollut 223 (3): 1045-1054.

Hidayat A. Tachibana S. 2013. Degradation of 2, 4, 8-trichlorodibenzoofuran by a new isolate of Cerrena sp. F0607. Int Biodeterior Biodegrad 77: 51-55.

Jarvinen J, Taskila S, Isomäki R, Ojamo H. 2012. Screening of white-rot fungi manganese peroxidases: a comparison between the specific activities of the enzyme from different native producers. AMB Express 2 (1): 62.

Jellison J, Jasalavich C. 2000. A review of selected methods for the detection of degradative fungi. Int Biodeterior Biodegrad 46 (3): 241-244.

Kachlishvili E, Metreveli E, Eliaishvili V. 2014. Modulation of Cerrena unicolor laccase and manganese peroxidase production. SpringerPlus 3 (1): 463.

Lee JS, Lim YW. 2010. Cerrena aurantiopora sp. nov. (Polyporaceae) from eastern Asia. Mycologia 102 (1): 211-216.

Leonowicz A, and Grzywaczewicz K. 1981. Quantitative estimation of laccase forms in some white-rot fungi using syringaldazine as a substrate. Enzyme Microbial Technol 3 (1): 55-58.

Lonergan GT, Jones CL, Mainwaring DE. 1993. The effect of temperature and culture medium on the degradative activity of Phanerochaete chrysosporium evaluated using three qualitative screening methods. Int Biodeterior Biodegrad 31 (2): 107-114.

Machado KMG., Matheus, DR, Bonomi VLR. 2005. Ligninolytic enzymes production and Remazol Brilliant Blue R decolorization by tropical Brazilian basidiomycetes fungi. Braz J Microbiol 36 (3): 246-252.

Moslanen U, Osma JF, Winquist E, Isolaila M, Couto SR. 2010. Decolorization of simulated textile dye baths by crude laccases from Trametes hirsuta and Cerrena unicolor. Eng Life Sci 10 (3): 242-247.

Mou DG, Lim KK, Shen HP. 1991. Microbial agents for decolorization of dye wastewater. Biotechnol Adv 9 (4): 613-622.

Rodríguez-Couto S. 2017. Industrial and environmental applications of white-rot fungi. Mycosphere 8 (3): 456-466.

Ryvarden L. 1984. Type studies in the Polyporaceae 16. Species described by J.M. Berkeley, either alone or with other mycologists from 1856 to 1886. Mycotaaxon 20 (2): 329-363.

Sadikin PV, Ariffin HS, Pramudya B, Mulatsih S. 2017. Carrying capacity to preserve biodiversity on ecotourism in Mount Rinjani National Park, Indonesia. Biodiversitas 18 (3): 978-989.

Sari AA, Tachibana S, Murayanto. 2012. Correlation of ligninolytic enzymes from the newly-found species Trametes versicolor U97 with RBBR decolorization and DDT degradation. Water Air Soil Pollut 223 (9): 5791-5792.

Septiningsrum K, Pramuji I. 2017. Aplikasi enzim di industri pulp dan kertas: I. Bidang pulp. J Selulosa 7 (1): 1-16. [Indonesian]

Somulashvili G, Spinder D, Jimenez-Tobon GA, Jaspers C, Kerns G, Penninckx MJ. 2015. Production of a high level of laccase by submerged fermentation at 120-L scale of Cerrena unicolor C-139 grown on wheat bran. CR Biol 338 (2): 121-125.

Suharyanto, Kresnawaty I, Prakoso HT, Eris DD. 2012. Aktivitas ligninolitik Omphalina sp. hasil isolasi dari TKKS dan aplikasinya untuk dekolonisasi limbah kosmetik. Menara Perkebunan 80 (2): 48-56. [Indonesian]

Sumandono T, Saragh H, Migrin, Watanabe T, and Amrita R. 2014. Decolorization of Remazol Brilliant Blue R by new isolated white-rot fungus collected from tropical rain forest in East Kalimantan and its ligninolytic enzyme activity. Procedia Environ Sci 28: 45-51.

von Rentelen K, Arida E, Häuser C. 2017. A review of biodiversity-related issues and challenges in megadiverse Indonesia and other Southeast Asian countries. Res Ideas Outcomes 3: e02860. DOI: 10.3897/tio.3.e20860

Warishu H, Valli K, Gold MH. 1992. Manganese (II) oxidation by manganese peroxidase from the basidiomycete Phanerochaete chrysosporium. J Biol Chem 267 (33): 23688-23695.

White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR Protoc 18:315-322.

Wulandari FY, Ratnamingsiya N, Dewi RS. 2014. Dekolorisasi limbah batik menggunakan limbah medium tanam Pleurotus ostreatus pada waktu inkubasi yang berbeda. Scripta Biologica 1 (1): 71-75. [Indonesian]

Yang J, Lin Q, Ng T B, Ye X, Lin, J. 2014. Purification and characterization of a novel laccase from Cerrena sp. HYB07 with dye decolorizing ability. PLoS ONE 9 (10): e110834. DOI: 10.1371/journal.pone.0110834.

Yanto DHY, Hidayat A, Tachibana S. 2017. Periodical biostimulation with nutrient addition and bioaugmentation using mixed fungal cultures to maintain enzymatic oxidation during extended bioremediation of oily soil microcosms. Int Biodeterior Biodegrad 116: 112-123.