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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. *Fauliana 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 (GRNP) 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 (GRNP), which composted 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; Suharyanto et al. 2012; Yanto et al. 2017; Rodríguez-Couto 2017). Other applications in biotechnology are for food, biosensor, biopulping, biobleaching, and biofuel (Septiningrum and Pramuaji 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 asl., 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'-azino-bis(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 ºC 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 ºC, 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 Taq® 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 phylogenic tree was constructed with maximum likelihood method using MEGA 7 software.

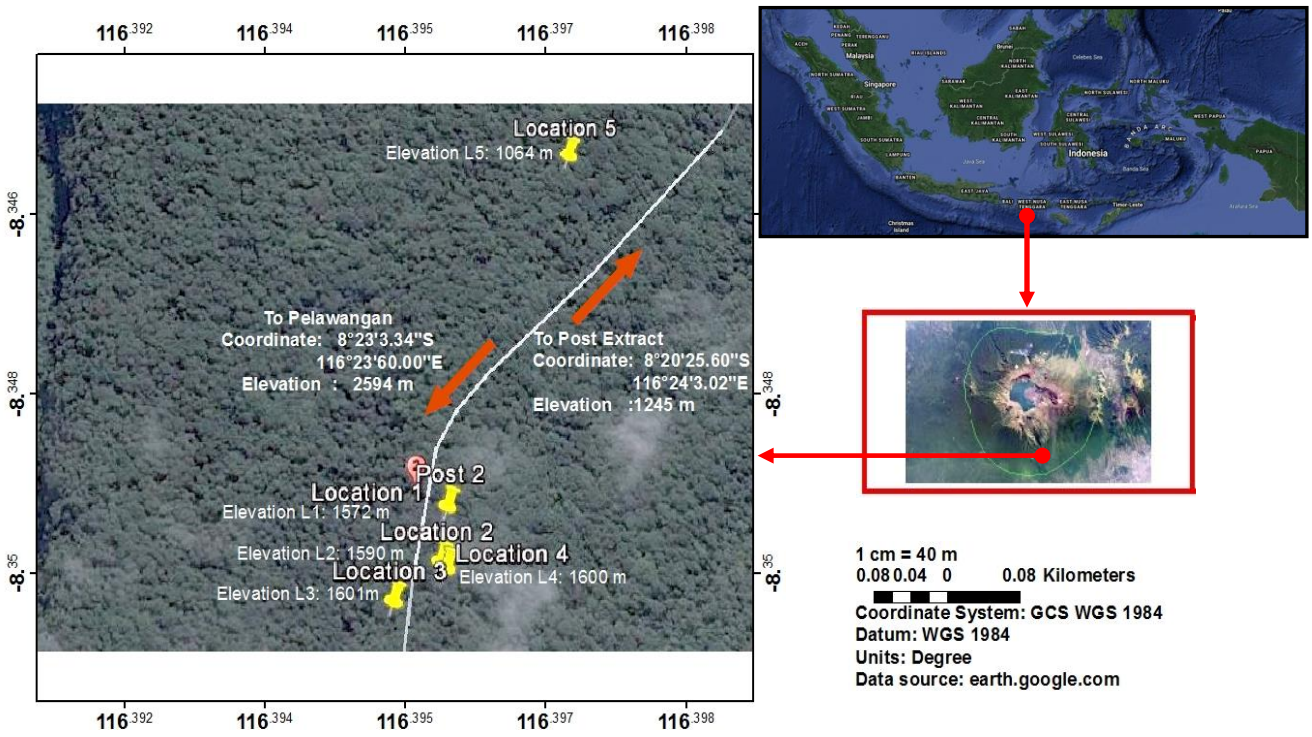

Figure 1. Map of sampling location in Gunung Rinjani National Park, West Nusa Tenggara, Indonesia

RBBR decolorization by *Cerrena* sp. BMD. TA. 1

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:

$$\% \text{ Decolorization} = [1-(C/C0)] \times 100\%$$

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 ($MnSO_4$) at 470 nm (Wariishi 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.

RESULTS AND DISCUSSION

Selection and identification of fungi

Total of 18 fungal strains isolated form GNRP were capable to grow and decolorize (disappearance of blue color) RBBR added to agar medium. The RBBR is a dye substrate that is commonly used as an indicator of ligninolytic enzyme activity, providing an easy method for selecting fungi that produce these enzymes (Lonergan et al. 1993; Sumandono et al. 2014). Previously reported *T. hirsuta* D7 produced lignolytic enzyme system and decolorized RBBR (Yanto et al. 2017), hence was used as a positive control in this study. Compared to *T. hirsuta* D7, the 18 fungal strains showed various decolorization capacities. Four fungal isolates (RJ005, TB.KC.2, KR.M.1, and BMd.TA.1) showed the most extensive RBBR decolorization zone (Figure 2).

Semi-qualitative and quantitative assays were performed on biochemical characteristics. The semi-qualitative assay was done with specific substrates; DMP, gallic acid, or syringaldazine, added to agar culture. The fungal isolates have shown different ability that was assessed by the ratio of color appearance and mycelium growth (less or more than 100%, Figure 3). Three isolates have been confirmed producing the high enzymatic activities for both laccase and MnP activity, compared to control isolate Figure 4, which were then subjected for subsequent quantitative assay using UV-Vis spectrophotometer. The result showed that strain BMd.TA.1 produced the highest level of both laccase and MnP activity (Figure 4). Consequently, strain BMd. TA. 1 was selected for further analysis. ITS region of isolate BMd.TA.1 genome was successfully amplified. Sequence of 615 base-pair long was aligned with NCBI database using BLASTn. The fungal isolate BMd.TA.1 showed high similarity (100% identity) with *Cerrena* sp. HYB07 partial sequence of small-subunit rRNA with accession number KX599411 (Figure 5).

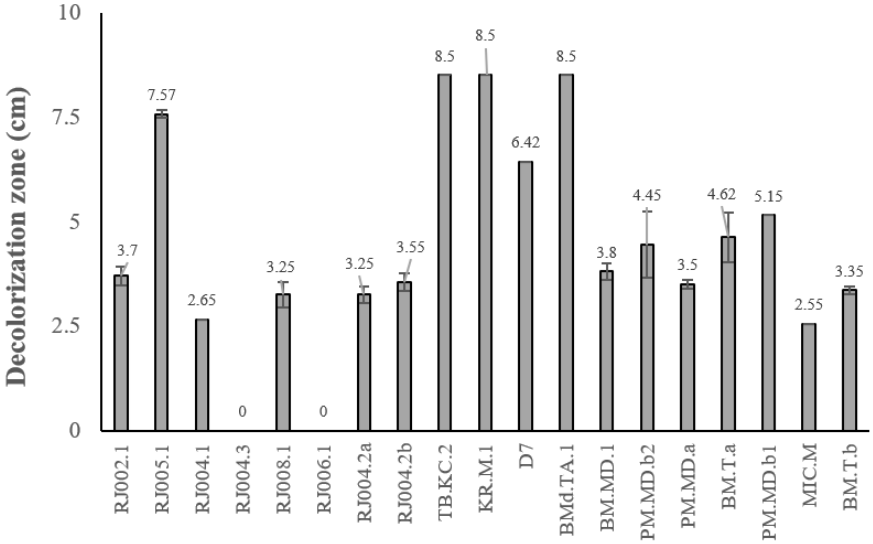

Figure 2. Decolorization of RBBR by 18 pure fungi and 1 fungal (D7) as the control on agar medium observed at 7 d

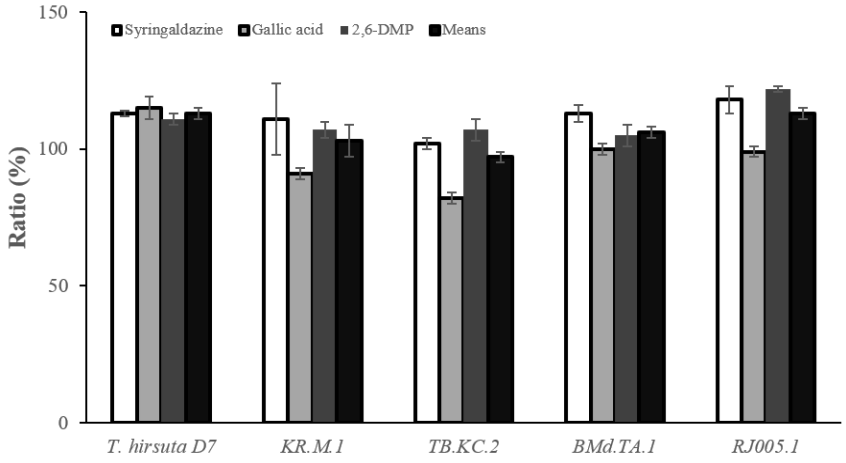

Figure 3. The effects of various substrated on ratio of decolorization and fungal growth. Mean of ratio is the average of quantitative value of all substrates. Syringaldazine, Gallic acid, 2,6-DMP were measured at 6 d, 4 d, and 5 d, respectively

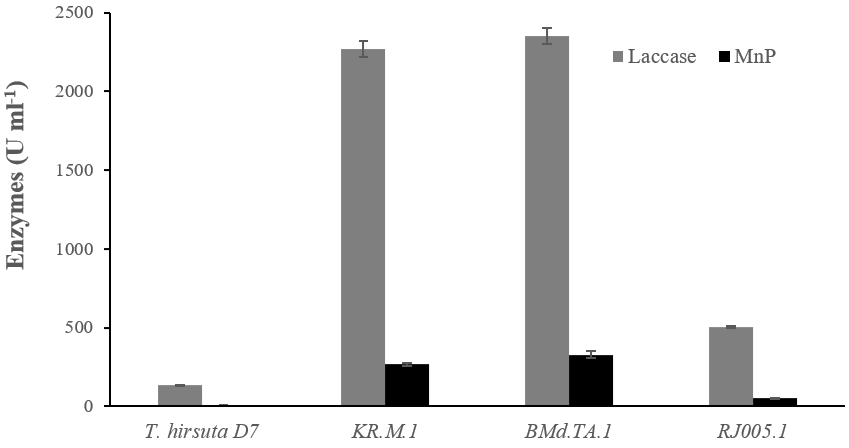

Figure 4. Laccase and MnP activity at liquid culture after 7 d

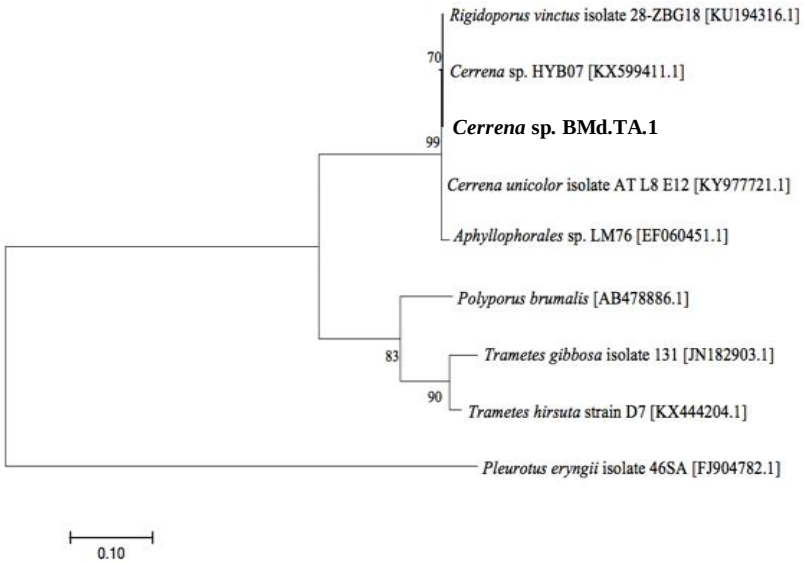

Figure 5. Phylogenetic relationship of *Cerrena* sp. BMd.TA.1 and related species sequences. The numbers in parentheses are NCBI accession number

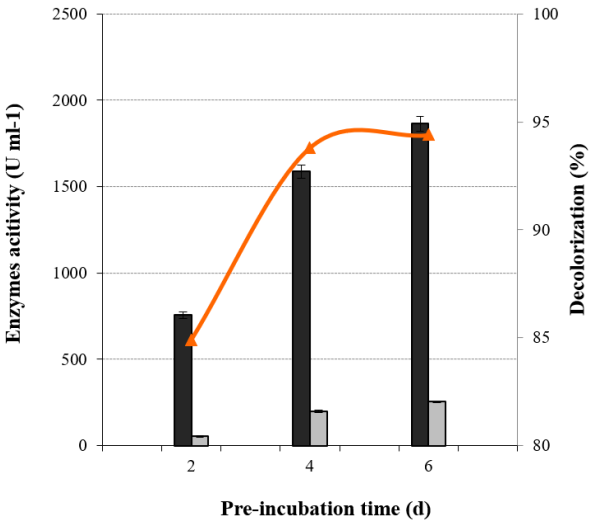

Figure 6. Effect of pre-incubation time on RBBR decolorization (▲), and Laccase (black bars) and MnP (grey bars) enzyme activity during the decolorization process by *Cerrena* sp. BMd.TA 1 in liquid culture

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} (Sonulashvili 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 ligninolitic 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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