

Bioprospecting three newly isolated white-rot fungi from Berbak-Sembilang National Park, Indonesia for biodecolorization of anthraquinone and azo dyes

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Abstract. Nurhayat OD, Ardiati FC, Ramadhan KP, Anita SH, Okano H, Watanabe T, Yanto DHY. 2022. Bioprospecting three newly isolated white-rot fungi from Berbak-Sembilang National Park, Indonesia for biodecolorization of anthraquinone and azo dyes. *Biodiversitas* 22: 613-623. Extensive use of textile dyes without proper wastewater treatment may jeopardize the water environment. In this study, bioprospecting newly isolated white-rot fungi from Berbak-Sembilang National Park for decolorizing four synthetic dyes was investigated. A total of 108 wood-decaying fungi were screened by using selective media, resulting in three isolates as the most promising fungal strains (BRB 11, BRB 73, BRB 81). BRB 81 had the best ability to decolorize 91.4% of AB129 and 77.8% of RB5 within 96 hours while the highest removal of RBBR and AO7 was performed by BRB11 around 60% and 37.6%, respectively. The enzymatic degradation was assumed to involve the decolorization process as laccase activities were observed with the highest around 116 UL⁻¹. Based on molecular identification, these three fungal isolates were identified as *Phellinus noxius* BRB 11, *Ceriporia lacerata* BRB 81, and *Leiotrametes menziesii* BRB 73, respectively. In conclusion, *P. noxius* BRB 11, *L. menziesii* BRB 73, and *C. lacerata* BRB 81 could be used as biological agents in textile wastewater treatment and thus, it is important to conserve them as a part of the biodiversity within the local biosphere reserve.

Keywords: *Ceriporia lacerata* BRB 81, decolorization, *Leiotrametes menziesii* BRB 73, *Phellinus noxius* BRB 11, synthetic dye

Abbreviations: RBBR: Remazol Brilliant Blue R; AB129: Acid Blue 129; AO7: Acid Orange 7; RB 5: Reactive Black 5; ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline)-6-sulphonate; MEA: malt extract agar; MGP: malt extract-glucose-polypeptone; WRF: white-rot fungi; LMEs: ligninolytic enzymes

INTRODUCTION

Synthetic dyes are regularly used in textile industries. In Indonesia, the textile industry is one of the largest foreign exchange earners and it had export valued at \$13 billion in 2019. This industry is one of the country's economic drivers, which contributes to employing more than 3 million people (Jakarta Globe 2019). However, its activities dispose of a large amount of water released to the environment, discharging colored wastewater around 40-65 L per kg of fabric resulting from the coloring process. The wastewater consists of synthetic dyes with different concentrations, depending on the group of the synthetic dye molecules structures (Imran et al. 2015). The release of this pollutant into water bodies without proper treatment will damage the aquatic environment, reducing photosynthetic activities due to the obstruction of sunlight into the water. These phenomena will decrease the water's dissolved oxygen levels, which may harm the entire water organism (Slama et al. 2021). The textile dyes are toxic and carcinogenic agents (Zhuo et al. 2019; Köktürk et al. 2021). Besides, the discharge of this pollutant could lead to a biomagnification effect (Newman 2015).

Several physical, physicochemical, and chemical methods, have been used to remove dyes from textile effluents. However, these methods produce hazardous secondary-product residual and relatively costly methods (Wang et al. 2020). Therefore, it is necessary to develop green technology to remove this pollutant to protect the aquatic environment. Currently, biological agents method has been seen as a cost-effective, energy-efficient, and environmentally-friendly approach (Lellis et al. 2019).

Bioremediation technique using white-rot fungi is an alternative way for textile wastewater treatment. The method has been famous for its cost-effectiveness and besides, the fungi can be found in all environments due to their fast adaption and metabolites diverse sources of carbon and nitrogen (Singh 2017; Zahmatkesh et al. 2018; Silva et al. 2018). Previous studies reported some white-rot fungi species such as *Phanerochaete chrysosporium*, *Ceriporiopsis subvermispora*, *Ganoderma* sp., *Leiotrametes flavidus* Zul62 can degrade synthetic dyes (Sumandono et al. 2015; Falah et al. 2018; Sudiana et al. 2018). Moreover, some studies demonstrated ligninolytic enzymes are produced by white-rot fungi could decolorize some anthraquinone and azo dyes, such as RBBR, AB129, AO7,

and RB5 (Anita et al. 2019; Yanto et al. 2019). The developed technique to immobilize these enzymes using some materials also showed a good rate of decolorization of synthetic dyes (Shaheen et al. 2017; Anita et al. 2020; Tišma et al. 2020; Yanto et al. 2021).

Laccases (EC 1.10.3.2) are extracellular multi-copper ligninolytic enzymes containing four copper ions, except for *Phlebia radiata* [Fr. (1821)] which has two copper ions discovered in fungi, bacteria some insects, and plants. This enzyme is very valuable for the application in various industries such as textile, food, pulp and paper, pharmaceuticals, cosmetics, and paint or furniture industry and within the bioremediation process (Mate and Alcalde 2017; Lellis et al. 2019; Song et al. 2020). Laccase has more potential advantages in the green biotechnological processes compared with other ligninolytic enzymes, such as manganese peroxidase (MnP) and lignin peroxidase (LiP). Laccase is an extracellular enzyme with low specificity that degrade several compounds with a phenolic structure, inducible, and does not need a cofactor for its activity (Plácido and Capareda 2015; Kumar and Chandra 2020). Due to its significant benefits, it is important to screen and isolate the laccase-producing fungal culture, especially from Indonesia's tropical forests, to enrich and diversify potential microbial culture collection.

Among different habitats, peat swamps forest has gained attention for its important roles in the carbon cycles on a global scale, especially as the significant carbon storage (Mary et al. 2011). Moreover, this habitat is rich in biodiversity (Page et al. 1997; Yule 2010) which act as valuable resources for diverse purposes (Pramudianto 2018). According to CIFOR (2021), Indonesia has the second-largest total wetland area globally, including the swamps and peatland. A previous research has successfully isolated a new fungus *Trametes hirsuta* D7 from peat swamp forest area in Riau to degrade phenanthrene, chrysene, and benzo[a]pyrene (BaP) (Hidayat and Yanto 2018). However, there are still limited studies and publications investigating microbial diversity and potency in the peat swamp forest in Indonesia, especially for dye decolorization. Therefore, this study aimed to evaluate the potential of newly isolated white-rot-fungi from Berbak-Sembilang National Park to decolorize synthetic dyes. The site is a part of the biosphere reserve designated by the United Nations Educational, Scientific, and Cultural Organization in 2018 under the Man and Biosphere Program (UNESCO 2019). The total area was around 3,819,837 which is dominated by undisturbed peat swamp forest in Berbak National Park while Sembilang National Park is well-known for its largest mangrove area within the Indo-Malayan region (RSIS 1991; RSIS 2012). The biodiversity is high with rare species of fauna and rich of palm species (UNESCO 2019) while the fungal species were rarely reported. The study results are expected to be beneficial to support the conservation of biosphere reserve, encourage sustainable use of biodiversity and act as an alternative way for wastewater treatment.

MATERIALS AND METHODS

Study site and sample collection

The study site was located in Berbak and Sembilang National Park, Jambi and South Sumatra Province, Indonesia. The fungal species used in this study were isolated from sporocarps collected from 4 locations in the Berbak-Sembilang National Forest, presented with the red dot in Figure 1. The sampling points consisted of peat swamp forests (points 1, 2, and 3) and mangroves area (point 4). All sporocarps samples were taken for further investigation at the Microbiology Laboratory, Research Center for Biomaterials, and Integrated Laboratory of Bioproducts (iLaB), National Research and Innovation Agency (BRIN), Bogor, Indonesia (Figure 1).

Procedures

Chemicals

RBBR, AB129, AO7, RB5, lignin, and ABTS were purchased from Sigma (USA). Table 1 shows the characteristics of four synthetic dyes used in this study. Polypeptone and chloramphenicol were purchased from Hi-Media (India). Agar, glucose, MEA, Dzapex-dox broth and other chemicals were provided by Merck (Germany).

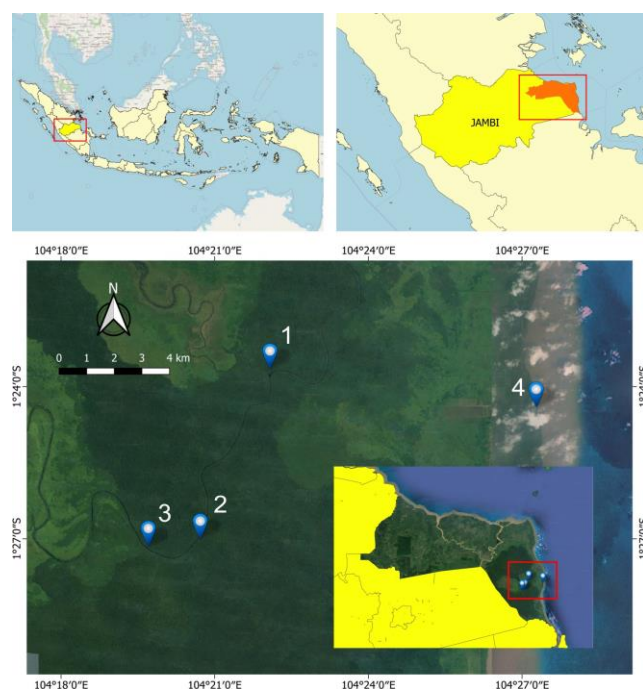


Figure 1. Location of Berbak-Sembilang National Park, the red dots are the sampling sites of fungus: point 1 is located in, Pondok Simpang Malaka (-1.39407, 104.36810), point 2 and 3 Simpang Gajah (-1.44999, 104.34540; -1.45238, 104.3285) and point 4 is located in Cemara beach (-1.406661, 104.45473)

Preliminary screening for ligninolytic enzyme-producer

The small pieces of internal sporocarps were isolated and cultured on alkali-lignin media that consist of KH_2PO_4 (1.5 g L^{-1}), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.3 g L^{-1}), KCl (0.3 g L^{-1}), NaNO_3 (3 g L^{-1}), yeast extract (0.3 g L^{-1}), malt extract (1.5 g L^{-1}), KOH (0.6 g L^{-1}), agar (30 g L^{-1}), lignin ($300 \mu\text{L}$), guaiacol ($60 \mu\text{L}$), chloramphenicol (0.75 g L^{-1}) at 25°C until mycelia growth was observed (Anita et al. 2011). The positive isolate was shown by the formation of a brown zone around the mycelia. Mycelia that grew from pieces of sporocarp was purified and cultured on MEA medium at 25°C for 7 days and maintained at 4°C .

Screening of new isolated white-rot fungus on selective media

All the positive fungi were inoculated into a Petri dish containing two layers of medium agar. The composition of the medium, bottom layer: Dzapek-Dox broth (35 g L^{-1}), KH_2PO_4 (1 g L^{-1}), yeast extract (2 g L^{-1}), polypeptone (2 g L^{-1}), agar 20 g L^{-1} , lignin (2 g L^{-1}), glucose (10 g L^{-1}); top layer: malt extract (5 g L^{-1}), chloramphenicol (0.4 g L^{-1}), RBBR (100 mg L^{-1}), agar (10 g L^{-1}). One plug ($\varnothing 5 \text{ mm}$) of 7 days old fungal colony growth on MEA agar was inoculated on top layers screening medium and incubated at 25°C for 7 days. Positive isolates were shown by forming a clear zone around the fungi growth (Oktaviani and Yanto 2015). The only positive isolate was screened based on the rate of culture growth and decolorization of RBBR on the two-layer medium for 7 days.

Decolorization of anthraquinone and azo dyes in the liquid medium

Three plugs ($\varnothing 5 \text{ mm}$) of 7 days old positive isolate growth on MEA agar were inoculated into 100 mL of Erlenmeyer flask with 40 mL malt extract-glucose-polypeptone (MGP) liquid medium that contains malt extract (20 g L^{-1}), glucose (20 g L^{-1}) and polypeptone (1 g L^{-1}). These were then incubated at 25°C for 7 days. After fungal biomass had formed, as much as $100 \mu\text{L}$ of each dye (RBBR, AB129, AO7, and RB5) was added into the culture so that the final concentration of dyes becomes 100 ppm culture medium. MGP liquid medium with the addition of dyes was used as a control. All reactions were performed in triplicate and incubated without shaking at 25°C for 96 h. Samples were taken with intervals of 24 h, 48 h, 72 h, and 96 h for decolorization and enzymatic assay.

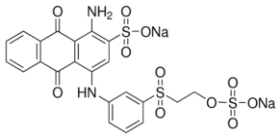
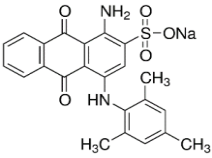
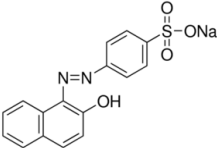
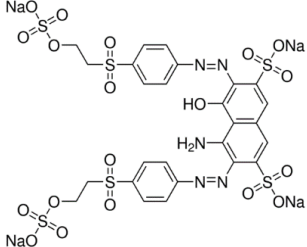
Decolorization assays

The decolorization process was measured at an interval of 24 h for 96 h and monitored at 592.5 nm for RBBR, 629 nm for AB129, 482.5 nm for AO7, and 598 nm for RB5 by UV-Vis spectrophotometer, UV-1800 Shimadzu, Japan. Decolorization was calculated by the following equation:

$$\text{Decolorization (\%)} = (A_i - A_f) / A_i \times 100$$

Where: A_i is the initial absorbance and A_f is the final absorbance of the mixture.

Table 1. Characteristics of synthetic dyes (RBBR, AB129, AO7, RB5)

Synthetic dye	Chemical structure	Functional group	Molecular weight
Remazol Brilliant Blue R		Anthraquinone dye	626.54
Acid Blue 129		Anthraquinone dye	460.68
Acid Orange 7		Monoazo dye	350.32
Reactive Black 5		Diazo dye	991.82

Enzyme assays

Laccase activity was observed spectrophotometrically by monitoring the oxidation of 2 mM 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) in 0.1 M acetate buffer pH 4.5 at 420 nm for 1 min. The assay mixture contained 100 µL of culture filtrate, 400 µL of 0.1 M acetate buffer and 500 µL of 2 mM ABTS. The following equation calculated enzyme activities:

$$\text{Enzyme activity (UL}^{-1}\text{)} = \left(\frac{\text{Abs.}}{\varepsilon} \times \frac{V_{\text{mixture}} (\mu\text{L})}{10^6} \times 10^6 \times \frac{60/t}{V_{\text{enzyme}} (\mu\text{L})/10^3} \right) \times 1000$$

Where *Abs* is the absorbance, ε is the molar absorptivity for laccase was 36,000 M⁻¹ cm⁻¹ (Irshad et al. 2011), *t* is time (min).

Identification of fungi

Fungal mycelia were grown on MEA at 25°C for 7 days. Mycelia were scrapped and put into a 1.5 mL plastic tube by using a scalpel blade. After the manufactured procedure, DNA was extracted using genomic DNA Mini Kit (Plant) (Geneaid Biotech Ltd., New Taipei City, Taiwan). Following DNA extraction and isolation, the internal transcribed spacer (ITS) region of the fungal nuclear rDNA was specifically amplified by KOD Neo Plus (Toyobo, JPN) and pairs of primers ITS 1F/ITS 4B (Gardes and Bruns 1993). Polymerase chain reactions (PCRs) condition was followed by PCR cycling as follows: 10 min at 95°C followed 30 cycles of denaturation 95°C for the 30s, annealing at 55°C for 30s, extension at 72°C for 60s, and a final extension at 72°C for 15 min, and then the temperature was held at 4°C for 10 min. Each PCR amplicon product was purified with FastGene Gel/PCR Extraction Kits (NIPPON Genetics, JPN) and sequenced using a 16 capillary 3130XL Genetic Analyzer (Thermo Fisher Scientific). The BigDye Terminator v3.1 Cycle Sequencing Kit was used with protocol by the manufacturer. PCR-amplicon products were sequenced in a forward and reverse direction using ITS 1F primer and the ITS 4B primer, respectively. Sequencing results were analyzed with taxonomic matches based on the BLASTN result with the highest sequence similarity (<http://blast.ncbi.nlm.gov/Blast.cgi>). The phylogenetic tree was constructed with the maximum likelihood (ML) method using MEGA 7 software (Kumar et al. 2016). The Bootstraps 1000 replication (BS) was used to test the strength of the internal branches of the phylogenetic trees (Felsenstein 1985). Other parameters used in the ML analysis were selected according to the default standard in MEGA 7 software. Bootstrap values of 50% or higher were shown.

Data analysis

All data were presented as the mean ± the standard error and calculated by using the Microsoft Excel program.

RESULTS AND DISCUSSION

Isolation and screening of fungi ligninolytic enzyme-producer

A total of 108 sporocarps of wood-decaying fungi were successfully collected from the sampling locations. Two selective media were used in the process of fungi isolates selection from those sporocarps that can decolorize synthetic dyes. In the first screening, qualitative assays were used to select the isolates that showed laccase activity on alkali-lignin media (Figure 2). A total of 22 isolates were able to grow in alkali-lignin medium but there were only 13 isolates that showed a reddish-brown zone around the fungal mycelia (Table 1). The reddish-brown zone around the fungal colony indicates, the ligninolytic enzyme, laccase, was produced to oxidize the lignin and guaiacol in alkali-lignin media during the growth of the colony (Thorn et al. 1996; Anita et al. 2011).

In the next screening, the positive isolates were grown in the agar medium containing lignin and RBBR synthetic dyes (100 ppm). Ramadhan et al. (2021) reported, the preliminary screening using two-layer agar media containing anthraquinone dye such as RBBR or AB129 on the top layer and lignin in the bottom layer effectively selected the promising white-rot fungi isolates for dye removal application. Among 13 isolates, only three promising isolates grew well and decolorized RBBR (Table 1), producing a clear zone around the mycelia (Figure 3). In this screening process, the lignin-containing in the medium will trigger the fungal isolate extracellularly produce the ligninolytic enzyme and oxidize the lignin. The previous study reported that lignin addition in media increased the fungal cell mass and ligninolytic enzyme activity of white-rot fungi (Thorn et al. 1996; Ferrara et al. 2002; Anita et al. 2011; Matjuškova et al. 2017). The ligninolytic enzyme extracellularly released by fungi in the medium then decolorized the RBBR dye via an oxidizing process (Moreira-Neto et al. 2013; Falah et al. 2018).



Figure 2. The reddish-brown zone around fungal isolate ligninolytic enzyme producer



Figure 3. Observation of three selected fungal isolates consists of BRB 11, BRB 73, and BRB 81. From left to right the pictures show: bottom view of the control plate, top and bottom view of the plate consists of three isolates. The samples were grown in RBBR-lignin agar medium during 7 days incubation at 25°C.

Table 1. Fungi isolate screening on alkali-lignin media and RBBR-lignin agar

Fungi isolate code	Alkali-lignin media	RBBR-lignin agar
	Reddish Brown zone (Oxidized lignin and guaiacol)	RBBR decolorization
BRB 5	-	-
BRB 11	√	√
BRB 12	-	-
BRB 16	-	-
BRB 24	√	-
BRB 28	√	-
BRB 29	√	-
BRB 36	√	-
BRB 37	√	-
BRB 38	√	-
BRB 39	-	-
BRB 46	√	-
BRB 50	-	-
BRB 53	√	-
BRB 57	√	-
BRB 58	√	-
BRB 72	-	-
BRB 73	√	√
BRB 74	-	-
BRB 76	-	-
BRB 81	√	√
BRB 104	-	-

Noted: √ = The positive isolate

Table 2. Growth rate and decolorization rate of three potential fungi isolates on RBBR-lignin agar

Isolate	RBBR-lignin agar	
	Growth rate (cm/days)	Decolorization rate (cm/days)
BRB 11	1.567 ± 0.004	1.608 ± 0.007
BRB 73	1.436 ± 0.004	0.799 ± 0.041
BRB 81	1.494 ± 0.006	0.603 ± 0.031

Three potential isolates (BRB 11, BRB 73, BRB 81) were then measured for their growth and decolorization rate on RBBR-lignin medium. However, the decolorization rate and mycelial growth rate varied for each fungal strain. The isolate BRB 11 had the most active isolate to grow and decolorize RBBR dyes in the medium compared to other strains (Table 2). Previous studies showed the growth rate

of fungal strain has a positive correlation to the decolorization activity (Kaur et al. 2015; Hefnawy et al. 2017; Ramadhan et al. 2021). However, in the case of fungal strain BRB 73 and BRB 81, the decolorization rate did not along with the growth rate. White rot fungal strains differ in their potential of dye decolorization because of the differences in their physiological characteristics to produce ligninolytic enzymes (Singh 2017; Yesilada et al. 2018). Due to these results, BRB 11, BRB 73, and BRB 81 were selected for the identification and decolorization of various synthetic dyes studies in liquid media.

Decolorization of synthetic dyes by three selected isolates

The decolorization assays in the liquid medium were performed by using the most promising fungal isolates. In general, increasing trends of decolorization percentage were observed by three isolates along with longer incubation time (Figure 4). Although the values were lower than 38% decolorization in 24 h for all dyes, the performance improved within 96 h. Among three fungal isolates, BRB81 had the best ability to remove AB129 up to 91.4% and RB5 with 77.8% removal while lower removals were observed for RBBR and AO7 as much as 51.4% and 13.47%, respectively. In contrast to BRB 81, the highest RBBR removal around 60% as well as 37.6% of AO7 removal were shown by BRB 11 but it had the lowest decolorization of 55.7% for AB129 and 21.5% for RB5. For strain BRB 73, it performed with the decolorization percentage in between of other two selected isolates with 50.6% removal of RBBR, 86.3% of AB129, 17.4% of AO7, and 51.3% of RB5. The maximum decolorization percentages showed a competitive performance compared to other studies. In the recent work by Ramadhan et al. (2021), three newly isolated WRF (*Trametes sanguinea*, *Neofomitella guangxiensis* and *Trametes polyzona*) could remove 59-89% of AB129 in 96 h which was slightly lower than the maximum removal by BRB81 (91%). The BRB 81 also showed a higher decolorization of RBBR dye (51%) compared to other *Ceriporia* sp. which had less than 40% removal within 96 h (Cerrón et al. 2015). In terms of AO7, which was known to be more difficult to be degraded, BRB11 could remove up to 37% that was higher than the removal by *T. versicolor* around 25-30% within 3 days (Yang et al. 2017) and other basidiomycetes fungi such as *C. versicolor*, *Pholiota* sp., and *Pleurotus* sp. which showed less than 20% removal (Nozaki et al. 2008).

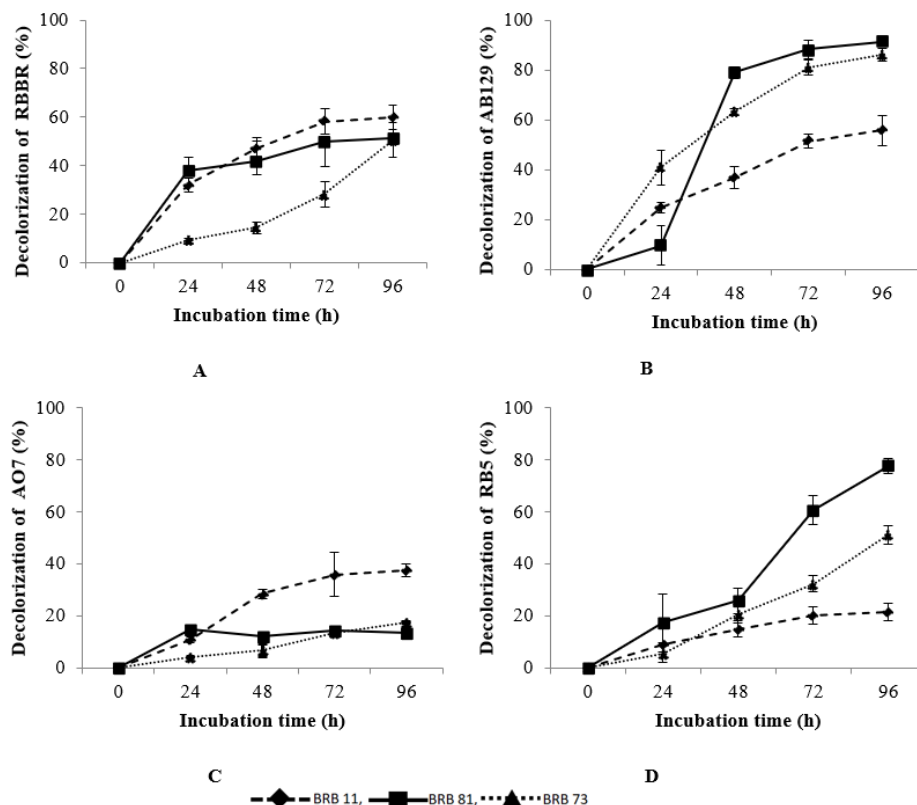


Figure 4. Decolorization effect on dyes when added to the 7 days old isolates. Decolorization of: A. RBBR; B. AB129; C. AO7; D. RB5

White-rot fungi have been extensively investigated for their potential to remove various dyes (Yesilada et al. 2018; Hanapi et al. 2018; Anita et al. 2019; Pecková et al. 2020). Based on the results, three selected isolates showed different abilities to remove different synthetic dyes. According to Yesilada et al. (2018), this phenomenon is possible due to the different physiological characteristics of each WRF fungal strain. Ramadhan et al. (2021) found similar findings, which studied decolorization by using *Trametes sanguinea*, *Trametes polyzona* and *Neofomitella guangxiensis*. The results showed that *T. polyzona* had the highest performance of 95% RB5 and 77% Acid Orange 7 removal while *N. guangxiensis* could only remove 20-22% of these dyes within 96 hours. Besides the type of strains, the predominant enzymes and their concentrations and the chemical structures and concentration of dyes could influence the decolorization percentage (Kale et al. 2014; Hanapi et al. 2018).

In this study, anthraquinone dyes were decolorized in a range of 50.6-60% and 55.7-91.4% for RBBR and AB129, respectively. On the contrary, azo dyes removals were observed between 21.5%-77.8% for RB5 and 13.5-37.6% for AO7 (Figure 4). Based on the average values between three fungal isolates within 96 h, anthraquinone dyes were easily removed compared to azo dyes. The results were persistent with previous studies which found anthraquinone dyes were decolorized more rapidly than azo dyes (Rodriguez et al. 1999; Champagne and Ramsay 2005; Zeng et al. 2011; Ramadhan et al. 2021). Yanto et al. (2019) also reported similar results and it was assumed that

the reasons might be due to stronger structural bonding of azo dyes compared to anthraquinone dyes. In addition, anthraquinone dyes were demonstrated as a substrate of laccase which enables direct oxidation for decolorization whereas azo dyes could not (Wong and Yu 1999). However, other factors which may impact the removal of the dye still should be considered such as dye structures, environmental conditions, and enzymes involved (Chulwan et al. 2004; Zeng et al. 2011; Yang et al. 2015).

Laccase assay by three selected isolates

Ligninolytic enzymes (LMEs) such as laccases, lignin peroxidases, and manganese peroxidases, produced by white-rot fungi have been famous for their ability to degrade lignin and other xenobiotic compounds, including dyes (Rodriguez et al. 1999; Couto 2009; Yesilada et al. 2018). Among these enzymes, laccases were the predominant enzymes involved in decolorization (Rodriguez et al. 1999; Champagne and Ramsay 2005; Birhanli and Yesilada 2006).

Furthermore, laccases synthesized by white-rot fungi commonly have high redox potential which could oxidize diverse molecules and be beneficial for higher efficiency of dyes removal (Yang et al. 2015; Legerská et al. 2016). Previous studies have been demonstrated the promising performances by laccases for decolorization of azo and anthraquinone dyes (Hadibarata et al. 2012; Cardoso et al. 2018; Anita et al. 2019). Therefore, this study focused on monitoring extracellular laccases activities.

At the initial phase, the highest enzyme activity was shown by BRB 11 for all synthetic dyes in a range of 54.47 to 116.3 UL^{-1} . On the other hand, BRB73 and BRB81 began with laccase activities varied between 7.1 to 24.25 UL^{-1} and 5.13 to 6.97 UL^{-1} , respectively. In comparison with previous studies, BRB11 and BRB73 secreted high maximum laccase activity during the decolorization, compared to the highest activity of: *T. polyzona* (40 U L^{-1}) within 3 days in RB5 and AO7 removals; *Ganoderma lucidium* (7.5 U L^{-1}) within 6 days in AO7 removals; *N. guangxiensis* (30 U L^{-1}) within 3 days in RBBR removal; *T. hirsuta* (21 U L^{-1}) within 1 day in AB129 removal (Ramadhan et al. 2021; Lai et al. 2017; Alam et al. 2021). The results were pertinent to previous studies, showing that laccase production might be varied among the fungal strains (Osma et al. 2011; Risdianto et al. 2012; An et al. 2020). Moreover, culture conditions and other factors which may impact the expressed enzyme activity should be considered (Rogalski et al. 1991; Merino-Restrepo et al. 2020). Along the incubation time, decreasing trends of laccase activity were observed for three isolates, except in RB5 by BRB11 (Figure 5). BRB11 and BRB73 showed a significant reduction of laccase activities within 96 hours while relatively stable enzyme activities were performed by BRB81. In contrast with a study by Rodriguez et al. (1999), it seems that there was no clear correlation between laccase activity with dyes decolorization such that BRB81 showed the highest decolorization with a relatively lower laccase activity. It might be that the incubation time was shorter within 96 hours, compared to previous studies that we're able to observe higher peaks within 9-20 days (Jaramillo et al. 2017; Merino-Restrepo et al. 2020).

Laccases (EC 1.10.3.2, *p*-diphenol: dioxygen oxidoreductase) belong to oxidative enzymes, catalyzing the oxidation of suitable substrates molecule to reactive radical and simultaneously reducing oxygen to water. In some cases, laccases require a redox mediator to oxidize the substrates with high redox potential or unsuitable specificity (Riva 2006; Legerská et al. 2016). However, direct oxidation of the dyes was able to occur in this study although the addition of enzyme mediators might enhance the decolorization (Yanto et al. 2019). Synthetic dyes degradation by white-rot fungi might occur through enzymatic degradation by LMEs, biosorption onto the surface of microbial biomass and/or in living cells, or the combination of both mechanisms (Kaushik and Malik 2009; Huang et al. 2016; Pecková et al. 2020). Based on the results, laccase activities were observed along with the removal of dyes (Figure 5), assuming enzymatic degradation contributed to the decolorization process. However, the sorption processes of the dyes onto the surface of fungal biomass might also occur. Many researchers have been reported the possibilities such as removal of RB5 by *Pleurotus eryngii* [(DC.) Quél. 1872] (Hadibarata et al. 2013), methylene blue by *Daedalea africana* and *Phellinus adamantinus* (Sintakindi and Ankamwar 2020) and congo red by *Ceriporia lacerata* [Suhara et al. (2003)] (Wang et al. 2017). Therefore, further investigation on the sorption capacity, decolorization by using purified laccases and or monitoring other LMEs can be done to understand the full potential of the isolates.

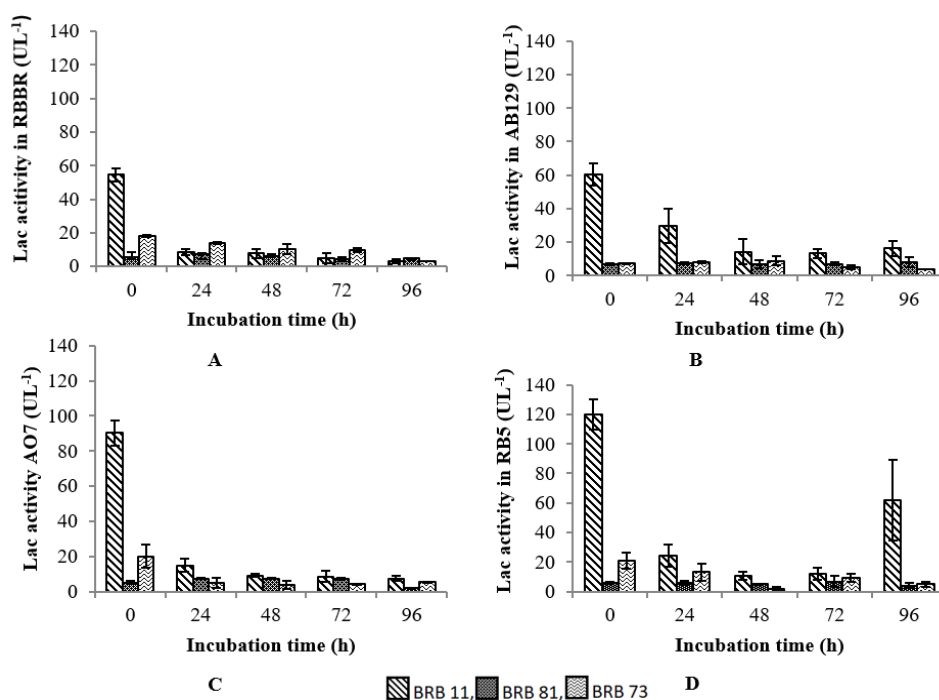


Figure 5. Laccase activity during decolorization process on 100 ppm of dyes (A) RBBR, (B) AB129, (C) AO7, (D) RB5 by three potential fungi isolates

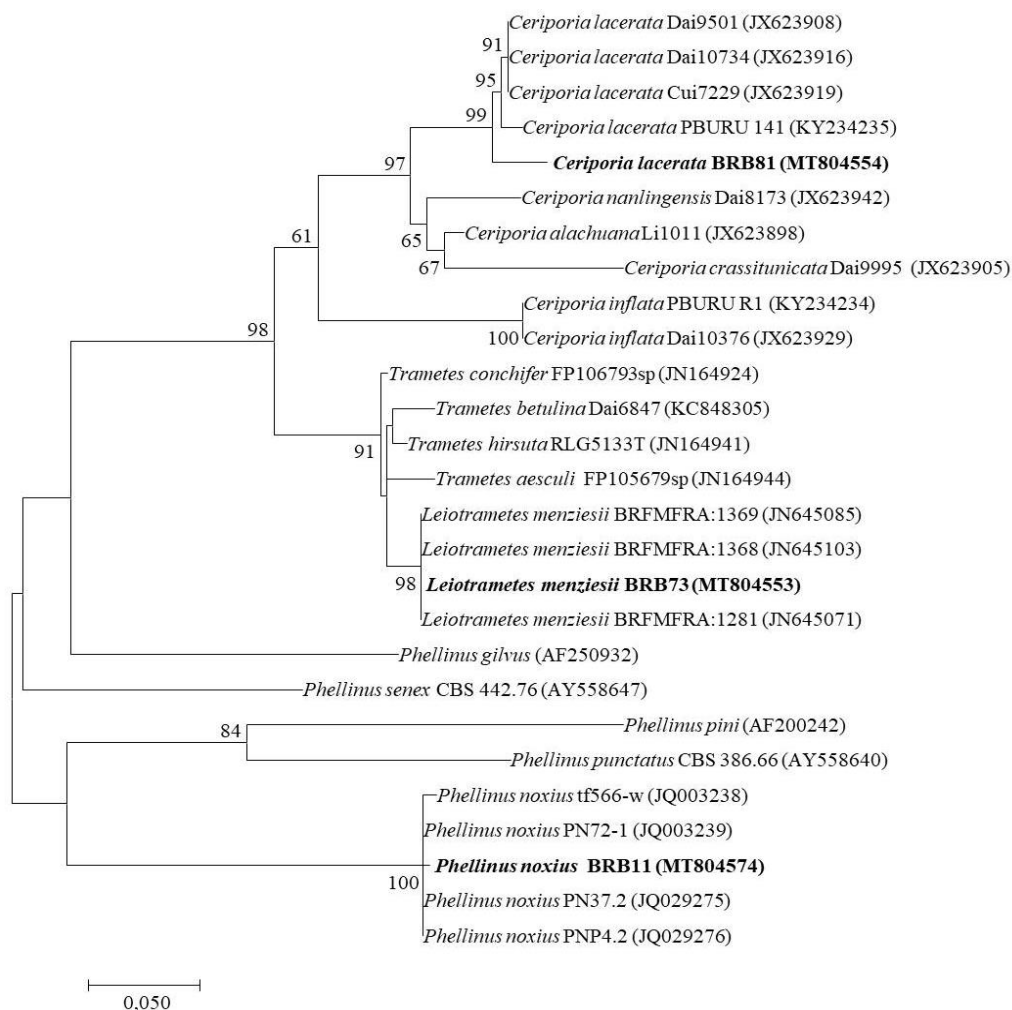


Figure 6. Phylogenetic tree isolates BRB 11, BRB 73, BRB 81, and related species based on ITS rDNA sequences. Bootstrap values > 50% are indicated at the nodes (1000 replication). The scale bar refers to an evolutionary distance of 0.050.

Identification of fungi

Phylogenetic tree analyses were carried out to identify three potential isolates by comparing their ITS rDNA sequences to their closest species from NCBI using the Maximum likelihood method. Moreover, all ITS rDNA sequence from those new isolates has been deposited to the NCBI database. BRB 11, BRB 73, and BRB 81 have accession numbers MT804574, MT804553, and MT804554 respectively (Figure 6). Based on molecular analysis of phylogenetic tree, BRB 11 isolate was *Phellinus noxius* with 100% bootstrap value. *Phellinus noxius* (Corner) Cunningham, is a white-rot fungus species known to cause brown root rot disease to the tree plant. This fungus species can be found in tropical and sub-tropical regions in Southeast and East Asia, Oceania, Australia, Central America, and Africa (Ann et al. 2002; Brooks 2002; Akiba et al. 2015). There were still limited studies about the decolorization process using *P. noxius*. We only found Poojary et al. (2012) that reported *P. noxius* hpf 17 able to decolorize more than 80% of textile dye such as Direct 7'C' Red (DR), Inco LiF Blue GLL-'C' (IBLue), Inco LiF Black (IBlack), Inco Fast Orange SE 'C'

(IFO), Chrysosphenine CH (CCH), Light fast brown BYRL (LFB), Inco swiss pink (ISP), Inco LIF yellow 54 LL 'C' IY, and Inco LiF Grey 2 RL 'C' (IGr). Accordingly, our study is the second report, that demonstrated the dye decolorization ability of *P. noxius*.

Next, BRB 73 isolate was identified as *Leiotrametes menziesii* BRB 73 based on 98 % bootstrap value of molecular analysis. The species of *L. menziesii* (Berk.) Welti & Courtec is a member of the novel genus *Leiotrametes* Welti & Courtec. Gen. Nov. This white-rot fungus has been distributed in Neotropical and Paleotropical regions (Welti et al. 2012). The scientific report of *L. menziesii* as a biocatalyst agent is still limited. Zhou et al. (2015) investigated the ligninolytic enzyme such as laccase and peroxidases of *L. menziesii* during solid-state fermentation in wheat straw and miscanthus. However, there were no scientific reports on the decolorization assay using this fungus species. Therefore, in this present study, the ability of *L. menziesii* BRB 73 was successfully demonstrated to decolorize several synthetic dyes.

The BRB 81 isolate was nested in the same clade to *Ceriporia lacerata* with 99% BS. Thus the BRB 81 isolate was identified as *C. lacerata* BRB 81. The first record isolation of this fungus in Indonesia was found at Samarinda Botanical Garden (KRUS), East Kalimantan and it was identified as *Ceriporia lacerata* PBURU 141. The result of maximum likelihood tree showed BRB 81 isolate clustered in the same position with *Ceriporia lacerata* PBURU 141 (KY234235). Furthermore, in terms of decolorization, recent studies reported the ability of this fungus species for decolorization purposes. This fungus was reported able to decolorize above 70% orange G dye via degradation mechanism by the enzyme in high salinity conditions (Chen et al. 2011). Whereas, Wang et al. (2017) reported the capability *Ceriporia lacerata* ZJSY of decolorizing congo red through mycelia absorption and degradation by ligninolytic enzyme.

To our knowledge, this is the first investigation on isolation, screening, and application of new isolates *P. noxius* BRB 11, *L. menziesii* BRB 73, and *C. lacerata* BRB 81 from Indonesia to removal various synthetic dyes. Therefore, in this study, it demonstrated a new bioprospection of Indonesia white-rot fungus isolated from Berbak and Sembilang National Park as biocatalyst for bioremediation purposes for wastewater containing synthetic dyes.

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