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Decolorization of Remazol Brilliant Blue R by laccase of newly isolated *Leiotrametes flavida* Strain ZUL62 from Bangka Heath Forest, Indonesia

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Abstract. *Falah S, Sari NM, Hidayat A. 2018. Decolorization of Remazol Brilliant Blue R by laccase of newly isolated* Leiotrametes flavida *Strain ZUL62 from Bangka Heath Forest, Indonesia. Biodiversitas 19: 633-639.* The use of microbial enzymes seems to be an appropriate approach to neutralize synthetic dyes, providing an eco-friendly and cost-competitive alternative solution for treating industrial effluents. The objective of this research was to select the most potential fungal isolate with high laccase activity to decolorize Remazol Brilliant Blue R (RBBR) synthetic dyes. The isolation of fungal body and sampling of wood decay specimens were conducted in six different locations in heath (kerangas) forest of Bangka Belitung, Indonesia. In the present study, a total of 13 isolates were screened by using indicator plate method and for their enzyme activity. The preliminary screening was done to screen ligninolytic fungi. Then the primary screening using various indicator compounds was conducted to select laccases-producing fungi. The enzyme activity assay was performed to select fungal isolate with the highest activity. We found that the most potential fungi belonged to *Leiotrametes flavida* Strain ZUL62, which had been confirmed by molecular identification using 5.8S rDNA/ITS analysis. In addition, the laccase from *Leiotrametes flavida* Strain ZUL62 could decolorize RBBR, exhibiting a high rate of decolorization rate of 62% without any mediator within 24 h of incubation. To our best of knowledge, this study represented the first report about *Leiotrametes flavida* Strain ZUL62 and its potential laccase enzyme for dyes effluent treatment.

Keywords: Decolorization, Leiotrametes flavida Strain ZUL62, laccase, RBBR, screening

Abbreviations: RBBR: Remazol Brilliant Blue R, ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline)-6-sulphonate, PDA: potato dextrose agar, PDB: potato dextrose broth, SDZ: syringaldazine, GA: gallic acid, h: hour, d: day, GA: gallic acid, SDZ: syringaldazine, MEA: malt extract agar, EC: Enzyme Commission

INTRODUCTION

Rapid industrialization has given rise to various unwanted elements that accumulated in the biosphere up to toxic levels and degrade the natural environment. Synthetic dyes are extensively used in diverse industrial applications ranging from food, pharmaceutical, paper printing, textile dyeing, color photography, cosmetic, and other industries. Most of them are highly toxic and recalcitrant pollutants. In addition, the effluents released into the environment accumulate as colored wastewater (Jin et al. 2007). This could lead to a serious problem for the ecosystem such as acute effects on exposed organisms due to the toxicity of the dyes; phytoplankton undergoing abnormal coloration and reduction in photosynthesis because of the absorbance of light that enters the water (Duran and Esposito 2000; Mester and Tien 2000; Chacko and Subramaniam 2011).

The color reduction of dyes in wastewater mainly based on physical and chemical methods. Although these methods demonstrate some good results, they have several drawbacks such as high cost, the formation of hazardous by-products, and high consumption of energy (Chulhwan et al. 2004). Sustainable development requires green technology from renewable sources for the production of environmental friendly products. Microbial treatment by enzymes are broadly used for hydrolysis of complex substrates and preferred to others because the biocatalyst works specifically to a particular substrate and can perform under much milder reaction conditions. Moreover, they do not produce undesirable products and hence eco-friendly. Previous reports demonstrated that some white rot fungi including *Armillaria* sp. F022 and *Cerrena unicolor* have the ability to degrade reactive black 5, Remazol Brilliant Blue R (RBBR), and Reactive Blue 4 of more than 50% after 4 to 6 d (Verma et al. 2010; Hadibarata et al. 2012). Furthermore, ligninolytic enzymes product by white-rot also showed the capacity to decolorize some anthraquinone and azo dyes, such as RBBR, Reactive Black 5, Congo Red, and Amaranth (Gavril et al. 2007; Garssi et al. 2011; Singh et al. 2011, Moilanen et al. 2010). The applications of these enzymes using immobilization to decolorize some dyes also demonstrated good degradation rates (Enayatimazir et al. 2011; Hidayat and Tachibana 2016).

Laccases (benzenediol: oxygen oxidoreductases;

Enzyme Commission (EC) 1.10.3.2) is the member of multi-copper enzymes of blue oxidases family which defined in the EC nomenclature as oxidoreductases that oxidize diphenols and similar compounds; thus, the enzymes have molecular oxygen as an electron acceptor (Thurston 1994; Viswanath et al. 2008). Laccases have been widely discovered in white rot fungi, bacteria, plants, and insects. Laccases are well known as "eco-friendly" enzymes due to its applications for bio-bleaching in pulp and paper industries (Fillat et al. 2010), wine and beer stabilization (Minussi et al. 2002), and biodegradation of textile dye and biosensor (Couto and Harrera 2006). As a result of the wide application of laccases in different biotechnological processes, there has been incredible scope for screening and isolation of potential microbes from Indonesia tropical forest for enrichment and diversity of laccase-producing cultures.

Heath forests are unique, and it is one of the rare tropical forest ecosystems. The heath or *kerangas* forest has been long known to exist in Kalimantan and Sumatra Islands. Heath forests are a type of seasonal lowland tropical rainforest that develop in dryland sites with predominantly podzolized, highly acidic, and sandy soils (Din et al. 2015). This study aimed to investigate a potential local isolate of white rot fungi, *Leiotrametes flavida* strain ZUL62, from Bangka heath forest in term of its capability to decolorize RBBR anthraquinone dye which is used in the textile industry and represents an important class of toxic and recalcitrant organopollutants.

MATERIALS AND METHODS

Sample collection

The fungal species used in this study were isolated from fungal bodies collected from six locations in the heath forest of Bangka Belitung province, Indonesia, presented with the yellow flags in Figure 1. All samples were isolated and cultured on malt extract agar (MEA) and taken for further examination at Forest Microbiology Laboratory, Forest Research and Development Center (FRDC), Research, Development and Innovation (FORDIA), Ministry of Forestry and Environmental, Bogor, Indonesia. Each sample was isolated and purified on the basis of its ability to decolorize RBBR.

Procedures

Chemicals

RBBR, 2,2'-azino-bis(3-ethylbenzothiazoline)-6-sulphonate (ABTS) was purchased from Sigma (USA). Agar, glucose, and all other chemicals were provided by Himedia (India) at the highest purity available.

Fungi, culture condition, and preliminary screening

The pure strains were cultured on MEA medium containing malt extract (ME) (20 g L^{-1}), glucose (20 g L^{-1}), agar (20 g L^{-1}), and polypeptone (1 g L^{-1}) at 25°C for 7 d and maintained at 4°C. Each isolate was screened based on the rate of culture growth and rate of decolorization of RBBR in agar medium for 7 d.

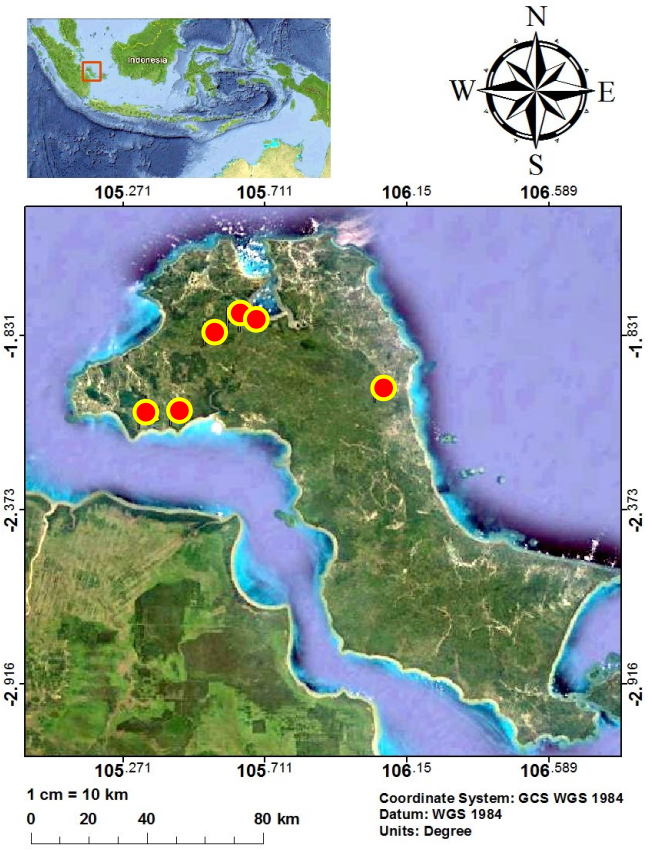

Figure 1. Map of sampling location in heath forest of Bangka region, Indonesia

Screening for fungal laccase-producer

The further screening was conducted with two different screening steps. The first screening was carried out by inoculation of mycelium from each strain onto potato dextrose agar (PDA) plates containing 0.1% gallic acid (GA), 0.04% ABTS, and 0.025% syringaldazine (SDZ), and incubated at temperature 30°C for 4 d. The quantitative value was determined by the measurement ratio of color clearance and mycelium growth. The second screening was the enzymatic assay of laccase activity. Three fungi isolate with the highest ratio of color clearance and mycelium growth from the first screening was selected, and cultured in PDA at 30°C. All selected fungi were cultivated in 50 mL potato dextrose broth (PDB) in 100 mL Erlenmeyer flask. Incubation was carried out at temperature 30°C for 7 d. The liquid culture from each sample was used for the enzymatic assay.

Identification of fungi

The fungal strains were identified using internal transcribed spacer (ITS region) of ribosomal RNA genes, amplified by polymerase chain reaction (PCR) using ITS1 and ITS4 primers and Go *Taq* Green Master Mix (Promega, USA) (Kumar and Shukla 2005). Genomic DNA was extracted using DNA Wizard Kit (Promega, USA) according to the recommended protocol. Confirmed PCR products were then Sanger-sequenced (First Base Sequencing Service, Singapore) and compared to database with National Center for Biotechnology Information

(NCBI)-BLAST (www.ncbi.nim.nih.gov). The phylogenic tree constructed with maximum likelihood method using MEGA 7 software (Kumar et al. 2016).

Enzyme purification

Leiotrametes flavida Strain ZUL62 was cultivated in sago dregs and PDB (1: 5). Crude enzymes were obtained by extraction of sago dregs fungus culture after 4 d incubation according to the method described by Hidayat and Tachibana (2016) with slight modification. The extraction was carried out by grinding using mortar with sodium acetate buffer (pH 5) addition, centrifuged at 10000 rpm, 4 ºC for 20 min. The supernatant obtained was added with ammonium sulfate (60%, v/w), and then centrifuged at 10000 rpm, 4ºC for 30 min. Precipitant was collected and added to acetic buffer. This solution was kept in refrigerator at 4ºC prior to use.

Enzymatic assay

Laccase activity was determined according to method described by Perez and Jeffriez (1992). The mixture containing 100 µL of 0.5 M sodium acetate buffer (pH 5), 20 µL of1 mM ABTS, and 40 µL enzyme solution was incubated at 30 ºC for 10 sec. ABTS oxidation was monitored by the increase in absorbance at 420 nm and read by the nano-spectrometer for 15 min. Laccase was expressed in international units (U) as 1 µmol of substrate oxidized per minute under the assay condition.

Experimental decolorization of RBBR

RBBR decolorization assay was performed by using nano-spectrophotometer with a final reaction volume of 160 µl at 595 nm wavelength. The reaction mixture for dye decolorization consisted of an aqueous solution of RBBR and purified laccase in sodium acetate buffer (pH 5). The RBBR solutions consisted of two concentrations, 100 and 1000 ppm. All the reactions were performed in triplicate and incubated without shaking at 40ºC with interval time 3 h, 6 h, and 24 h. The decolorization percentage was calculated by the following equation:

$$\% \text{ Decolorization} = (1-(At_{595}/Ao_{595})) \times 100\%$$

Ao and At refer to the initial and final absorbance at 595 nm, respectively.

Data analysis

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

RESULTS AND DISCUSSION

Isolation and screening of fungi laccases-producer

Of the 22 fungi samples, there were only 13 isolates obtained as pure strains. The pure strains were continuously evaluated in agar medium containing RBBR (100 mg L^{-1}). Five of the isolates grew well as indicated by the covering of color change of RBBR of more than 95% on the petri dish (diameter = 8.5 cm) (Figure 2.A). Those isolates were B20A (100%), B8B2 (95%), ZUL62 (95%), B11D (100%), and B20C (100%). In the next screening, the selected isolates were screened by qualitative assay. Figure 2.B shows the growth ratio of the five fungi on agar medium containing GA, ABTS, and SDZ. Based on these assays, we obtained 3 promising fungi isolates. In order to select the fungus with high laccase production, the three isolates were further evaluated using laccase enzymatic assay. The laccase activity of the three isolates is shown in Figure 2.C. The highest laccase activity was exhibited by ZUL62 (22.58 ± 2.54 U mL^{-1}), followed by B20A (13.95 ± 4.12 U mL^{-1}), and B8B2 (10.77 ± 2.19 U mL^{-1}). Thus, ZUL62 was the most promising isolate and selected for further study.

Identification of fungi

Phylogenic tree was constructed based on the ITS sequence (ITS1/ITS4) of isolate ZUL62, and 11 reference strains analyzed by BLAST using the maximum likelihood method and MEGA 7 program (Figure 3). The 619 bp ITS sequence (ITS1/ITS4) of isolate ZUL62 has been deposited at the NCBI database with accession number MF774416. The NCBI BLAST online search tool showed that the partial ITS sequence of isolate ZUL62 had a maximum similarity index of 99% relative to strains of *Leiotrametes* sp. The phylogenetic tree showed the isolate ZUL 62 was closely related to *Leotrametes flavida* (Accession No. KC589131.1 and KC589130.1); and thus, the isolate was named *Leiotrametes flavida* strain ZUL62 (Accession No. MF774416).

Decolorization of RBBR by purified *Leiotrametes flavida* Strain ZUL62 laccase

The purified *Leiotrametes flavida* Strain ZUL62 laccase (the activity = 0.015 U μL^{-1} with the yield = 8.05%) was able to decolorize RBBR (Figure 4). A maximum absorbance of RBBR was detected at 595 nm, and from this peak, the absorbance decreased with the increase of incubation time (3, 6 and 24 h). This result revealed that oxidation had occurred by laccase enzyme. However, the decolorization seemed to be influenced by concentration of RBBR. At low concentration of RBBR (100 ppm), the dye was decolorized by 62% in 24 h (Figure 4). The treatment of laccase also decolorized higher concentration of RBBR (1000 ppm), but the decolorization rate was 17% lower than that of the 100 ppm.

Discussion

The term 'heath forest' is used to describe a tropical rainforest comprising less tall trees with smaller leaves in comparison with those of the more widespread Tropical Lowland Evergreen Rain Forest (Whitmore 1984). The study at heath forest has been focused on environmental condition (before and after mined land), diversity of vegetation, and its correlation with soil properties (Din et al. 2015; Oktavia et al. 2015). However, there has been less study on the exploration of the potential microbial component at heath forest, including white rot fungi laccase-producer.

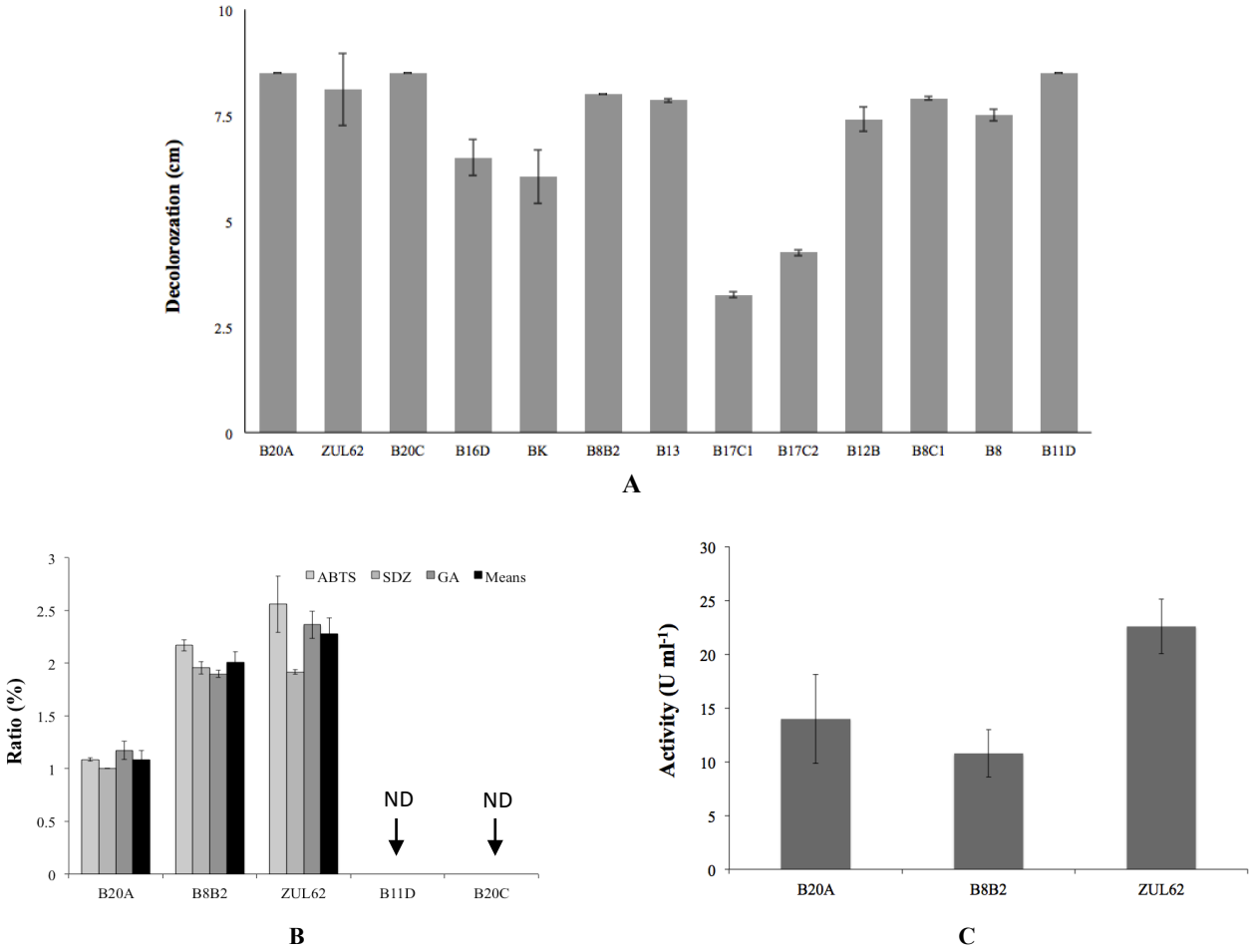

Figure 2.A. Preliminary screening of fungi using RBBR agar medium; B. Screening of fungal ligninolytic enzymes producer, ND = not detected; C. Screening of the most fungal laccase-producer

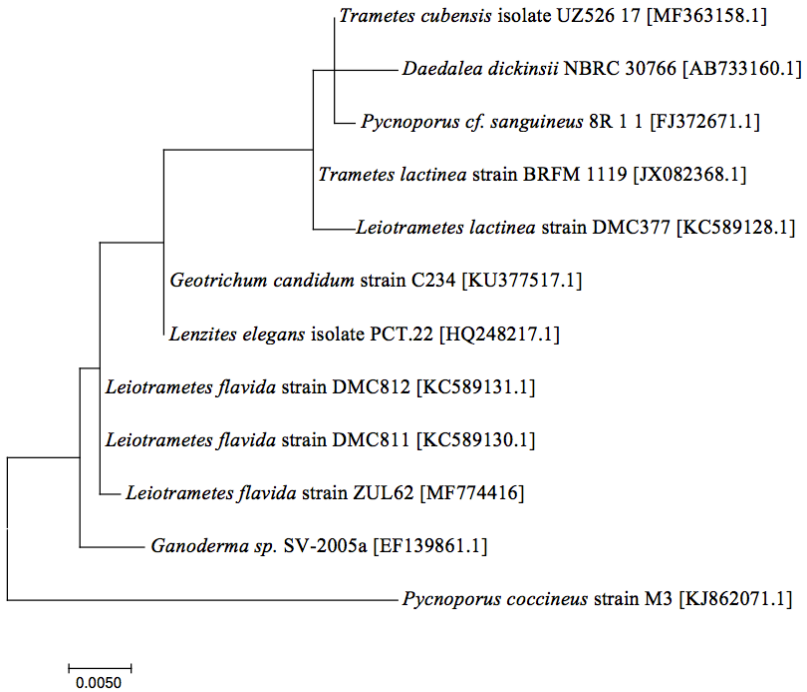

Figure 3. Phylogenic tree of isolate *Leiotrametes flavida* Strain ZUL62. The accession numbers for the NCBI database are shown in parentheses

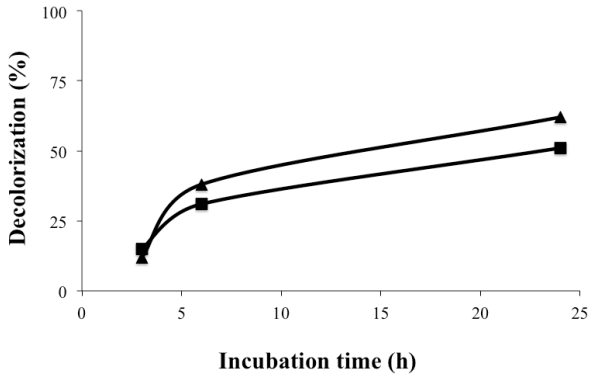

Figure 4. Decolorization rate of RBBR by purified laccase isolated from *Leiotrametes flavida* Strain ZUL62, with 100 ppm (▲), and 1000 ppm (■) of RBBR concentration

In this study, various fruiting bodies of fungi that grow well predominantly in decaying wood at heath forest were collected from several locations in Bangka and West Bangka district, Bangka Belitung province (Figure 1). They were isolated to obtain pure strain and screened with three steps process. Later, *Leiotrametes flavida* strain ZUL62 was selected as a potential laccase producer. This isolate demonstrated an RBBR-decolorization rate of more than 95% (Figure 2.A). This approach was a preliminary screening, and similar methods can be developed for other objective such as determining fungi having the ability to break down some recalcitrant organic compounds (Passarini et al. 2011; Yanto et al. 2017). Fungi capable of degrading RBBR as substrates in the selection process will produce some enzymes that have a role in decaying woods, lignin and cellulose degradation (Wunch et al. 1997). The further screening showed that isolate ZUL62 had the best growth performance in agar medium containing GA, ABTS and SDZ followed by a high level of laccase production (Figure 2.B and 2.C). The use of GA, ABTS, and SDZ in the screening process has been previously reported (Lonergan et al. 1993; Hidayat and Tachibana 2013). Production of laccase in those three indicators could be seen clearly. The dark-brown color would have appeared when GA is present, dark-purple is the indicator color for ABTS, and purple-pinkish-colored for SDZ (Cavallazzi et al. 2005; Wang et al. 2010; Kumar et al. 2011; Chan et al. 2016; Sivakumar et al. 2010; Vantamuri and Kaliwal 2015; Alfarra et al. 2013). Hidayat and Tachibana 2013 reported that *Cerrena* sp. F0607 produced laccase when isolated and screened using the same method used in this study (Hidayat and Tachibana 2013). Many studies supported that Genus *Cerrena,* but not *Leiotrametes flavida* could produce a high level of laccase (Verma et al. 2010; Desai and Nityanand 2011),.□

The activity of laccase during 7 d incubation of ZUL62 in liquid culture was 22.58 ± 2.54 U mL^{-1} . Several studies reported that the laccase activity during incubation in liquid medium showed various results, i.e., *Cerrena* sp. F0607 (0.14 U U mL^{-1}), *Trametes versicolor* U97 (1.2 U mL^{-1}), *T. pubescens* MB.89 (2 U mL^{-1}), *C. unicolor* (0.7 U mL^{-1}), and *Fomes sclerodermus* (9.9 U mL^{-1}) (Hidayat and Tachibana 2013; Sari et al. 2012a; Papinutti et al. 2003; Kachlishvili et al. 2014; Galhaup and Haltrich 2003); thus, placing our ZUL62 isolate as among the best fungus in term of the production of laccase. Furthermore, the activity of the purified ZUL62 laccase obtained in this study (59.3 U mL^{-1}) was higher than that of the WRF-1 laccases (30.07 U mL^{-1}) obtained from another previous study (Mishra et al. 2011). Laccase is capable of catalyzing the oxidation of a broad range of substrates, and applied to wide purposes including decolorization of wastewater dyes (Hamedaani et al. 2007; Hidayat and Siregar, 2017). Among all dye types, anthraquinone dyes are the second widely used synthetic dye in the world; but it has potentially high resistant to degrade due to its aromatic structure (Banat et al. 1996). The decolorization of RBBR, an anthraquinone dyes, was evaluated through the application of the purified laccase from Isolate ZUL62. We found the decolorization of RBBR was about 62% in the initial substrate concentration of 100 ppm within 24 h. Furthermore, other studies showed several results for decolorization of RBBR as follow: (i) *T. versicolor* U97 fungal culture was about 50% and 85% in 96 h and 144 d, respectively (Sari et al. 2012b); (ii) Purified laccase of *Arthrospira maxima* was about 49% in 96 d (Afreen et al. 2017); (iii) Purified laccase of *Armaliraia* sp F022 was about 70% in 48 h (Hadibarata et al. 2012); (iv) purified laccase of *Paraconiothyrium variabile* was about 47% in 3 h by addition of laccase mediator (Forootanfar et al. 2012). The increasing of RBBR concentration resulted in the reduction of decolorization (Figure 4). The reduction of decolorization level was caused by the high dye concentration, implying the less average attacks of enzyme to each dye molecule, and hence slower color clearance rate (Young and Yu 1997). The processes for decolorization of dyes are affected by various parameters, such as pH, temperature, initial dyes concentration, dye types, mediator, and enzyme types (Kaushik and Malik 2009; Hadibarata et al. 2012). This study concluded that *L. flavida* strain ZUL62, a new fungus isolate from heath forest of West Bangka district, Bangka Belitung province was capable of producing high level of laccase. The RBBR decolorization obtained was 95% and 62% by agar culture method and purified laccase activity, respectively. *L. flavida* strain ZUL62 is a highly potential fungus for biological treatment of colored effluent for anthraquinone dyes through laccase catalysis.

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