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Activity test on laccase enzyme of fungus *Volvariella volvacea* for decolorization of remazol reactive dye

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Abstract. *Sari TP, Susilowati A, Setyaningsih R. 2022. Activity test on laccase enzyme of fungus* Volvariella volvacea *for decolorization of remazol reactive dye. Asian J Trop Biotechnol 18: 73-78.* Textile industry waste such as remazol reactive dye is a source of pollution that can damage aquatic ecosystems. This waste is difficult to degrade because it has strong covalent bonds. However, this compound can be degraded biologically (decolorization) using the laccase enzyme oxidoreductase. This study aimed to determine the ability of the laccase enzyme produced by the fungus *Volvariella volvacea* (Bulliard ex Fries) Singer to decolorize remazol reactive dye. The test begins with the decolorization of the dye by the fungal culture of *V. volvacea*, the production and purification of the laccase enzyme with ammonium sulfate, and the decolorization test with the purified laccase enzyme. The results showed that the fungal culture of *V. volvacea* was able to decolorize remazol reactive dye on agar and liquid medium enzymatically. In addition, based on a statistical analysis of ANOVA followed by a *Post-Hoc Test* DMRT method test level of 5%, the results showed that the percentage of decolorization of the three dyes by the laccase enzyme, which had the highest enzyme activity (5.42 U/mL) from the ammonium sulfate purification increased along with increasing incubation time. The highest decolorization percentage occurred in remazol brilliant blue R (RBBR) with an incubation time of 48 hours.

Keywords: Decolorization, laccase enzyme, remazol reactive dye, *Volvariella volvacea*

INTRODUCTION

The textile industry in Indonesia is an industry that continues to grow from year to year. Based on data from Badan Pusat Statistik, in the first quarter of 2019, the growth of the textile and clothing industry was recorded at 18.98%, and this number increased compared to the profit during 2018 of 8.73% (Damayanti 2019). Textile industry waste has polluted the aquatic environment. On the island of Java, water pollution by the textile industry occurs in several watersheds, such as the Citarum River and the Bengawan Solo. Based on data compiled by Greenpeace Indonesia and the Institute of Ecology of Universitas Padjadjaran, it was stated that there was an increase in the concentration of pollutants whose values were higher than usual standards originating from approximately 800 textile factories operating around the Citarum River and the city of Majalaya (Birry and Meutia 2012). Another study around the Bengawan Solo watershed found that the wastewater produced by the textile industry had exceeded the standards of textile industry wastewater quality, namely BOD = 60 mg/L, and COD = 150 mg/L (Lolo and Pambudi 2020).

Most textile industry waste contains synthetic dyes, which are difficult to degrade due to their strong covalent bonds. One of the most frequently used dyes is azo dyes, such as reactive remazol (Kant 2012; Haryono et al. 2018). Azo reactive dyes (N = N) are of significant concern because they are precursors of dyes and form the most significant synthetic dyes with various colors and structures. Therefore, this dye is widely used in the textile industry (Kannan et al., 2013). However, due to the structural properties of aromatic amines, reactive dyes are not readily biodegradable under natural conditions and are not easily removed from waterways by conventional wastewater treatment systems (Babu et al., 2007; Lewis, 2014).

Most of the dye waste cannot be recycled. Still, it can be treated with several handling methods such as coagulation, membrane technology, flocculation, ozonation, Fenton reactive, reverse osmosis, cucurbutyril, electrochemical degradation, and activated carbon. The weakness of these methods lies in the costs involved because it needs many costs for each type of processing. Many textile industries do not treat their waste and throw it directly into waterways. On the other hand, the dye waste can cause turbidity, make the water look bad, smell bad, and prevent light penetration (Widjajanti et al., 2013). Therefore, many kinds of research on waste treatment have been carried out at a reasonably low cost and are easy to do, namely by bio-treatment or processing involving a specific organism (Babu et al., 2007; Sasmaz et al. 2011).

Using organisms, especially microorganisms, in degrading waste is an environmentally friendly alternative. Several microorganisms such as bacteria, fungi, yeast, and microalgae have been found to be able to remove textile dyes (Olukanni et al., 2006; Gowri et al., 2014). These organisms have developed enzyme systems for decolorizing and mineralizing reactive dyes, especially remazol, under certain environmental conditions

(Ekambaran et al., 2002; Faulina et al., 2020). In the case of reactive dye enzymatic remediation, laccase appears to be the most promising enzyme. Laccase has been shown to remove various industrial dyes by decolorizing or decreasing the intensity of the dye (Nyanhongoa et al. 2002).

Laccase enzyme (Lac) is an enzyme that belongs to the ligninolytic enzyme group along with other enzymes, namely manganese peroxidase (MnP) and lignin peroxidase (LnP) (Sharma et al. 2017). Each of these enzymes has the ability to degrade different dyes. Several studies on remazol reactive dyes have shown that high levels of laccase can degrade dyes efficiently (Novotny et al., 2004; Gowri et al., 2014; Sharma et al., 2017). Laccase enzymes are produced mainly by ligninolytic fungi such as *Phanerochaete chrysosporium, Trametes Versicolor, Pleurotusostreatus*, and *Volvariella volvacea* (Novotny et al. 2004; Devi et al. 2012). The fungus *V. volvacea* (Bulliard ex Fries) Singer belongs to the Basidiomycetes group, which usually produces several laccase isoforms. When grown in submerged culture under different conditions, *V. volvacea* produced at least two protein bands with laccase activity. Previous physiological studies have shown that laccase production by *V. volvacea* is induced by copper and various aromatic compounds (Chen et al. 2004a; Ahlawat and Kaur 2018; Akhlawat et al. 2008).

In this study, the activity of the laccase enzyme in the decolorization of remazol reactive dye will be tested using the fungal laccase enzyme *V. volvacea*. This research was carried out in several stages of testing, namely, decolorization of remazol reactive dye with a fungal culture of *V. volvacea* on agar and liquid medium, production and purification of laccase enzyme with ammonium sulfate fraction, measurement of enzyme activity with ABTS substrate, measurement of protein content using the Bradford method, and test decolorization ability of remazol reactive dye with the purified fungal laccase enzyme *V. volvacea*. This research is expected to provide more excellent opportunities in the textile industry to treat waste and reduce pollution.

MATERIALS AND METHODS

The materials used were white-rot fungus *V. volvacea* obtained from the CV. Volva Indonesia (Sleman, Yogyakarta), sterile distilled water, 70% alcohol, 0.5% sodium hypochlorite, potato dextrose agar (PDA), lactophenol cotton blue, $CuSO_4$, streptomycin, dye remazol brilliant blue R (RBBR), remazol black B (RBB), and remazol brilliant red (RBR), glucose, KH_2PO_4, $MgSO_4$, $CaCl_2$, yeast extract, thiamine, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) or ABTS, ammonium sulfate ($(NH_4)_2SO_4$), acetic acid (CH_3COOH) 0.2 M, sodium acetate (CH_3COONa) 0.2 M, $Na_2HPO_4.7H_2O$, $NaH_2PO_4H_2O$, trizma HCl, trizma base, bovine serum albumin (BSA), and Bradford's reagent.

Isolation of the fungus *Volvariella volvacea*

Fungal isolates were made using tissue culture techniques. The fruit bodies of the fungus *V. volvacea* that have been surface-sterilized are prepared. Then, the fruiting body is cut with a sterile knife, and the inside is taken thinly. Thin slices of fruiting bodies were planted in plates containing PDA media and incubated at 30ºC for seven days.

Decolorization test on agar medium

The decolorization ability test of the fungus *V. volvacea* in degrading dyes was carried out by testing its activity in PDA media which had added RBBR, RBB, and RBR dyes with a concentration of 100 ppm and CuSO4 inducer, respectively. Streptomycin was added to the media solution before being poured into Petri dishes (20 µg/1000 mL). 7 days old fungal mycelium agar blocks with a diameter of 0.5 cm were inoculated on the media incubated at 30ºC for 14 days. Colony growth and color fading on the media were observed every day.

Decolorization test on liquid medium

The decolorization test on liquid media was performed by mixing 100 mL of basal media with 100 ppm of dye and $CuSO_4$ as an inducer into a 250 mL bottle. The basal medium used consisted of (gL^{-1}) glucose-5, KH_2PO_4-1, $MgSO_4$-0.5, $CaCl_2$-0.14, Yeast extract-1, Thiamine-0.0025 (Jonathan et al. 2009). The media was inoculated with five pieces of agar mycelium of fungal culture on day 5 (diameter 0.5 cm) and grown under sterile conditions. The cultures were incubated in a shaker incubator at 110 rpm at 30°C (Gbolagade et al. 2006). After 72 hours, the cultures were taken and centrifuged at 5,000 rpm for 1 minute. The absorbance of the supernatant was measured using a UV-Vis spectrophotometer at the maximum wavelength of each dye sample. The result of the absorbance measurement is then calculated as the decolorization value with the following formula: [(A0–At/A0)] x 100, where A0 is the initial absorbance on the first day of inoculation and At is the absorbance at a specific time (Gomaa and Momtaz 2015).

Production of the laccase enzyme of fungus *Volvariella volvacea*

Enzyme production media consisted of 100 mL basal media, 100 ppm dye, 0.05 mM CuSO4, and streptomycin (20 µg/1,000 mL). The medium was inoculated with five pieces of agar mycelium of the fungus *V. volvaceae* and incubated with a shaker at 120 rpm, temperature 30°C, and for as long as nine days. The production medium was separated from the mycelium and centrifuged to obtain a crude extract of the laccase enzyme.

Enzyme purification with ammonium sulfate fractionation

Purification of the laccase enzyme was carried out by precipitation using ammonium sulfate fractionation. The crude extract of the laccase enzyme was precipitated with a certain degree of saturation (0-20%, 20-40%, 40-60%, and 60-80% (w/v)). The crude extract of the laccase enzyme

was added slowly with ammonium sulfate saturation of 0-20% and stirred for 30 minutes. After centrifugation, the enzyme precipitate and supernatant were obtained. The precipitate was dissolved in 100 mM acetate buffer pH 5, as much as 10% of the total volume (Vivekanan et al., 2014; Falah et al., 2018). The supernatant obtained was then precipitated by ammonium sulfate with saturations of 20-40%, 40-60%, and 60-80%. The Bradford method tested the precipitate results and then tested for protein content. Also, the enzyme activity was tested with ABTS as a substrate.

Optimization of temperature and pH for decolorization with purified laccase enzymes

The reaction mixture for decolorization temperature and pH optimization consisted of 500 L of dye with a concentration of 100 mg/L, 300 L of 100 mM acetate buffer pH 4.5, and 100 L of purified enzyme extract. Temperature optimization was carried out at temperature variations of 4°C, 30°C, and 60°C. While the pH optimization was carried out with variations of 100 mM acetate buffer pH 3 and 5, phosphate buffer 100 mM pH 7, and Tris-HCl buffer 100 mM pH 9. Temperature and pH optimization were carried out for 24 hours.

Dye decolorization test with purified enzymes

Decolorization testing using purified enzymes was based on variations in time and dye. The concentration of dye used is 100 ppm. Each reaction mixture was incubated for 3, 6, 24, and 48 hours. Samples were analyzed using a spectrophotometer with a maximum wavelength of each dye used. The maximum wavelengths of RBBR, RBB, and RBR dyes are 595 nm, 590 nm, and 600 nm, respectively (Gowri et al. 2014). After measuring with a spectrophotometer, the percentage value of decolorization was calculated.

RESULTS AND DISCUSSION

Dye decolorization on agar medium

Colony growth was observed to determine the toxic nature of the dye concentration used. In observations within 14 days, inhibition of colony growth only occurred at the beginning of the growth phase. This indicates a process of adaptation of fungi to the media with the addition of dyes. According to Tavcar (2006), the fungus will adapt to the environment in the decolorization process. After the adaptation phase, the enzyme will work by degrading the dye. The results of degradation are nutrients for fungal growth.

The color fading from the decolorization can be seen in the media's color change on the dish's back view. The decolorization process in this test involves the enzymatic mechanism of oxidative enzymes to oxidize the dye to fade. According to Singh and Singh (2010), the decolorization mechanism by fungi can be divided into 2 systems, namely enzymatic and non-enzymatic. The enzymatic mechanism occurs by secreting oxidative ligninolytic enzymes, especially laccase, to mineralize dyes and break down aromatic bonds in dye complexes, resulting in color fading. In contrast, the non-enzymatic mechanism is dye adsorption by the fungal mycelium (adsorption). In this study, the absence of mycelium color change in the fading dye medium indicated that the decolorization process with adsorption did not occur. Therefore, the decolorization process is possible due to the activity of ligninolytic enzymes, especially laccase.

Dye decolorization on liquid medium

The percentage of decolorization is increasing day by day with increasing incubation time (Figures 1-2). On day 15, the percentage of RBBR dye decolorization was 57%. RBB is 49%, and RBR is 45.19%. The dye was easily decolorized by the fungus *V. volvacea*, with the highest decolorization value being RBBR dye.

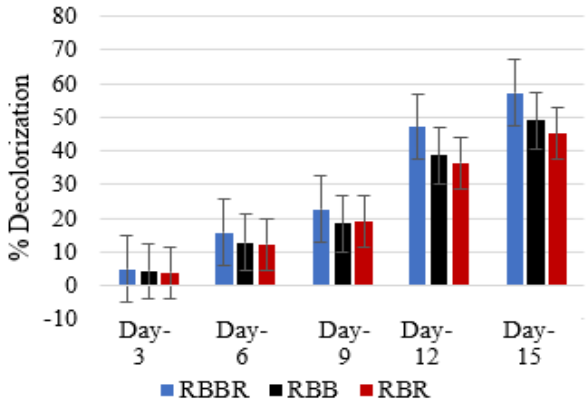

Figure 2. Percentage of decolorization of remazol brilliant blue R (RBBR), remazol black B (RBB), and remazol brilliant Red (RBR) by the fungus *V. volvacea* in liquid culture medium

Figure 1. Color fading by the fungus *V. volvacea* on day 14: A. RBBR, B. RBB, C. RBR

The decolorization process with the fungal culture isolates *V. volvacea* was influenced by the condition of the laccase enzyme, which was still maintained or not denatured by environmental conditions. According to Romero et al. (2006), applying decolorization using fungal culture causes ligninolytic enzymes such as laccase to be protected because they are associated with cells. The decolorization value can be seen from the change in the absorbance value. The change in absorbance value was caused by the dissolution of the dye chromophore group by the activity of oxidative enzymes such as laccase produced by the fungus *V. volvacea*. The laccase enzyme will break down large compounds in the dye into simpler ones with low molecular weights. In addition, the decolorization process is also influenced by the ability of fungal isolates in the adsorption process. According to Martani et al. (2011), fungi can decolorize dyes with non-enzymatic systems through the absorption of dyes by fungal mycelium. The addition of the $CuSO_4$ inducer also influenced the increase in decolorization percentage. According to several studies that have been conducted, $CuSO_4$ is the most commonly used and most effective inducer because the effect of copper produced affects increasing the secretion of the laccase enzyme (Gomaa and Momtaz 2015). The increase in decolorization of remazol dye with liquid culture fermentation was caused by the greater induced laccase enzyme. It was more active in decomposing the substrate, namely the dye (Haedar et al., 2019). According to Erkurt et al. (2007), the decolorization of RBBR dye was influenced by the laccase enzyme, which was induced in the presence of Cu^{2+} compounds in the culture medium.

Enzyme production and purification

The laccase enzyme production resulted in a crude extract with an enzyme activity of 3 U/mL. The production of the laccase enzyme uses a glucose carbon source. The fungus oxidizes glucose to oxalic acid, which accumulates in the medium. The decrease in glucose in the medium was accompanied by a buildup of oxalic acid and a decrease in pH. The extracellular enzymes secreted by fungi are too large to pass through the pores of the fungal cell wall. Calcium in the cell wall is bound by oxalic acid. It can further damage the integrity of the cell wall and provide an opportunity for extracellular enzymes such as laccase to accumulate in the medium (Munir et al., 2001; Bertrand et al., 2013).

To obtain a purer enzyme extract, the enzyme production was precipitated by ammonium sulfate fractionation. The enzyme activity obtained in the 0-20% fractionation was 1.21 U/mL with a total activity of 60.28 U. In the 20-40% fraction, the enzyme activity increased by 3.06 U/mL with a total activity of 137.7 U. For the 40-60% fraction, the protein precipitate was increasing, indicating that the interaction between the enzyme and ammonium salt was getting better. The resulting enzyme activity value was 5.42 U/mL with a total activity of 162.6 U. In the 60-80% fraction; the enzyme activity decreased by 2.17 U/mL with a total of 54.25 U (can be seen in Table 1). This shows that the interaction between the enzyme and the ammonium sulfate salt is reduced.

The protein content of the crude laccase extract to the highest fraction of 60-80% experienced a significant decrease. The initial protein content of the crude extract was 0.114 mg/mL with a total protein of 11.418 mg. The protein content in the purification fraction 0-20% was 0.0649 mg/mL with a total protein of 3.2447 mg. In the 20-40% fraction, the protein content was 0.0271 mg/mL with a total protein of 1.2213 mg. In the 40-60% purification fraction, the protein content was 0.014 mg/mL with a total protein of 0.418 mg. Furthermore, the highest purification fraction was 60-80%, and the protein content was 0.0077 mg/mL with a total protein of 0.1915 mg. The higher the total protein sample, the lower the specific activity. This indicates more protein contaminants than the lactase enzyme, which interferes with substrate conversion to product. Increased protein enzyme purity also occurred in the fractionation with the highest laccase enzyme activity, which was 40-60%. The total enzyme protein in the 40-60% fraction was 0.4182 mg with a specific activity of 112.75 U/mg. In this fraction, the purity of the enzyme increased by 14.8 times with a yield percentage of 54.2% from the purity of the crude extract one time with a yield percentage of 100%. In another study conducted by Chen et al. (2004b), the laccase enzyme from *V. volvacea* isolate had the highest activity value at 80% ammonium sulfate saturation with a total enzyme protein of 7 mg and a specific activity of 3.6 U/mg. In this fraction, the purity of the laccase enzyme is 2.8 times, and the yield is 77% of the purity of the crude extract of the enzyme one time with a yield percentage of 100%.

Table 1. Test data on enzyme activity and protein levels of the fungal laccase enzyme *V. volvacea* purified ammonium sulfate

Fraction	Vol (mL)	Enzyme Activity (U/mL)	Protein (mg/mL)	Total Protein (mg)	Total enzyme activity (U)	Enzyme specific activity (U/mg)	Purity (times)	Yield (%)
Raw Extract	100	3,0000	0,1142	11,4188	300,0	26,2724	1,00	100,00
0-20%	50	1,2056	0,0649	3,2447	60,28	18,5779	0,71	20,09
20-40%	45	3,0600	0,0271	1,2213	137,7	112,7516	4,29	45,90
40-60%	30	5,4200	0,0139	0,4182	162,6	388,8401	14,80	54,20
60-80%	25	2,1700	0,0077	0,1915	54,25	283,2242	10,78	18,08

Table 2. Percentage of remazol reactive dye decolorization by the laccase enzyme of fungus *V. volvacea* in the variation of decolorization time

Dye type	Decolorization percentage on time variation (%)			
	3 hours	6 hours	24 hours	48 hours
RBBR	0,086a±0,075	5,903c±1,361	19,500g±1,290	20,316g±1,007
RBB	2,746ab±1,000	6,483cd±0,991	14,127ef±1,688	15,040f±1,435
RBR	4,450bc±0,667	8,357d±1,189	12,740e±1,846	15,960f±1,576

Dye decolorization with purified enzymes

The optimum temperature for decolorizing remazol reactive dye is 30°C with a percentage of 15.11%. This is aligned with Qin et al.'s (2019) research, which states that the percentage of decolorization by the laccase enzyme of the fungus *Ganoderma lucidum* decreases in conditions below 20°C and above 50°C. In studies with other white-rot fungi, the laccase enzyme's decolorization temperature can be achieved at 40-70°C (Si et al., 2013; Patel et al., 2014; Yan et al., 2014). Based on Murugesan et al. (2009) research, the percentage of decolorization of RBBR dye can be achieved at a maximum temperature of 60°C; beyond that, it can decrease sharply. At the same time, the optimum pH for decolorization is pH 5, with a percentage of 14.86%. According to Si et al. (2013), *Trametes pubescens* laccase enzyme is active and stable at alkaline pH (pH 7.0 to 10.0). However, in another study, most white-rot Basidiomycetes were optimum at a pH between 2.2 to 5.0 (Patel et al., 2014; Yan et al., 2014). This indicates that laccases from different sources have different characteristics. The optimum temperature for decolorizing RBBR dye by fungal laccase *G. lucidum* was optimum at pH 4.0 (Murugesan et al. 2009).

Decolorization percentage data based on a variation of incubation time and remazol reactive dye were analyzed by ANOVA statistical test at a 95% confidence level ($\alpha = 0.05$). If $p < 0.05$ (significantly different), the DMRT further test was conducted at the 5% level. The highest decolorization percentage occurred in RBBR dye, with a decolorization time of 48 hours. Each dye has a different speed of decolorization time. However, the highest overall decolorization percentage value for each dye occurred at 48 hours of incubation (Table 2). Based on interval data from time to time variation, the decolorization percentage indicates the possibility of increasing decolorization after 48 hours. According to Yanto et al. (2019), the percentage of dye decolorization by the laccase enzyme increased with increasing incubation time. The decolorization process will stop after the laccase enzyme has finished reacting to the dyed substrate.

The value of decolorizing remazol reactive dye by purified laccase enzyme is low. In another study, the decolorization of RBBR dye (concentration 100 ppm) with the laccase enzyme from the purified Basidiomycetes isolate ZUL62 resulted in a decolorization value of 62% at a 24-hour incubation time (Falah et al. 2018). In addition, another study using the lactase enzyme from the fungus Trametes Versicolor U97 decolorized RBBR dye by 50% (Sari 2012). This study shows the low percentage of decolorization is caused by possible factors that interfere with the decolorization process. One of them is the low purity level of the laccase enzyme, causing low enzyme activity. In addition, the temperature instability of the purified laccase enzyme causes the enzyme to be easily denatured. According to research, Grassi et al. (2011) stated that the use of purified enzymes was susceptible to unstable conditions compared to the laccase enzyme's raw filtrate. Another possibility that can cause the low value of decolorization is the presence of decomposition products. Based on research by Romero et al. (2006), the product of decomposition in the decolorization process with purified enzymes can cause inhibition of dye degradation.

In conclusion, the laccase enzyme from the culture of fungus *V. volvacea* was able to decolorize remazol reactive dye on agar and liquid culture medium. Decolorization also occurred in the test with the laccase enzyme due to the precipitation of ammonium sulfate with an enzyme activity of 5.42 U/mL. Furthermore, each decolorization value increased with increasing incubation time. This indicates that the laccase enzyme from the fungus *V. volvacea* has the potential as a remazol reactive dye decolorizing agent.

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