JOURNAL OF DEGRADED AND MINING LANDS MANAGEMENT
Volume 10, Number 1 (October 2022):3829-3839, doi:10.15243/jdmlm.2022.101.3829
ISSN: 2339-076X (p); 2502-2458 (e), www.jdmlm.ub.ac.id

Research Article

Screening and identification of fungi isolated from batik wastewaters for decolorization of Remazol Black B dye and batik effluent

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Article history:
Received 8 June 2022
Accepted 26 July 2022.
Published 1 October 2022

Keywords:
Aspergillus
decolorization
fungi
laccase
Remazol Black B dye

Abstract

Azo dyes are the most commonly used dyes in the textile industry and is classified as reactive dyes, including remazol dyes. Remazol dye creates additional reactions with fibrous substrates to produce ester bonds that give the fabric a bright color. Remazol Black B (RBB) is a reactive dye from the Azo group. Remazol, also called a reactive vinyl sulfone dye which is widely used in the batik industry with black B, is its kind of color. One of the biological treatments uses bioremediation techniques using fungi as a bioremediation agent. Therefore, this study aimed to screen and identify potential fungi that could degrade RBB using tannic acid as a qualitative screening and quantitative screening using a liquid medium containing various concentrations of RBB dye (250 ppm, 500 ppm, 1000 ppm, 1500 ppm). The results showed among ninety-eight fungal isolates, and six isolates were positive for laccase assay using tannic acid. Two of the six fungal isolates were identified as *Aspergillus* sp.1 (74BRT) and *Aspergillus* sp.2 (105PDL), which were selected for further study based on their high efficiency in decolorizing RBB dye (96.89% and 91.21%). BLAST analysis of sequence data showed the identity of isolate 74BRT as *Aspergillus tamarii,* and isolate 105PDL as *Aspergillus sclerotiorum*. The efficiency of *A. tamarii* and *A. sclerotiorum* to decolorize the batik effluent was up to 37.47% and 42.09%, respectively. The laccase assay of these two isolates showed that *A. tamarii* had the highest enzyme activity at 120 h, reaching 12.23 IU $mL^{-1,}$ while *A. sclerotiorum* reached 9.34 IU mL^{-1}.

To cite this article: Ayu, Y.S. and Kasiamdari, R.S. 2022. Screening and identification of fungi isolated from batik wastewaters for decolorization of Remazol Black B dye and batik effluent. Journal of Degraded and Mining Lands Management 10(1):3829-3839, doi:10.15243/jdmlm. 2022.101.3829.

Introduction

The textile industry is one of the industries that release about 10 to 15% of dye which then enters wastewater (Selvam et al., 2003). The textile industry, such as batik, contributes to a large amount of waste that is disposed of during the dyeing process (Khalik et al., 2015). Azo dyes are the most commonly used dyes by the textile industry and are classified as reactive dyes, including remazol dyes. Remazol dye creates additional reactions with fibrous substrates to produce ester bonds that give the fabric a bright color (Solomon et al., 2009; Aryanti et al., 2020).

Some reactive dyes have a complex aromatic structure that makes them stable, so they are difficult to degrade in conventional effluent treatment (Kumari et al., 2016). These structures can make a dangerous risk when the chemicals in these reactive dyes accumulate. The structures that are not easily degraded naturally in naphthol and remazol dyes have been studied by various methods to degrade these dyes, for

example, dye degradation using electrochemical methods (Gupta, 2009). This method is effective in degrading the types of soluble and insoluble dyes by reducing the chemical oxygen demand (COD), but the main disadvantage of this method is that it consumes high electricity costs, produces sludge and pollution from chlorinated organics and heavy metals due to indirect oxidation (Rachmawati, 2016).

Biological methods by utilizing microbial activity can be an alternative solution in the management of batik dye waste. This method is more efficient because it is relatively simple, inexpensive, environmentally friendly, and does not produce secondary waste in the form of sludge which can become a new environmental problem (Kumari et al., 2016). One of the biological treatments is using bioremediation techniques, which means using a biological system to degrade toxic components in the environment (Dewi and Lestari, 2010). The study by Khalid et al. (2011) showed that isolated fungi strain S4 isolated from soil had the most effective in removing the Remazol Black B dye. More than 95% decolorization by the strain S4 was observed in soil under optimal incubation conditions. In a study conducted by Ning et al. (2017) *Aspergillus flavus* A5p1 was able to degrade Direct Blue 71 dye belonging to the azo dye group with a percentage of 76.4%, and in a study conducted by Ryu (1992), it was found that *Aspergillus sojae* B-10 was able to degrade the azo dye group up to 86%.

The purpose of this study was to determine the ability of potential fungal isolates to decolorize variations in concentrations of Remazol Black B dye and batik effluent in a liquid medium and to determine the activity of the laccase enzyme.

Materials and Methods

The sludge, effluent, and affected soil samples used for fungal isolations were collected from the local environment of batik effluent storage ponds at several home industries in Bantul, the Special Region of Yogyakarta. Indonesia. The reactive dye used in the study was Remazol Black B (RBB), obtained from one of the home industries of batik in Bantul, the Special Region of Yogyakarta. Indonesia.

The isolation of fungal from the sample was isolated using serially diluted up to 10^{-7}. Distinct fungal colonies were repeatedly sub-cultured until pure isolate was obtained and used for further study. Ninety-eight purified fungal isolates were screened for laccase production using tannic acid as a substrate. One plug (one cm diameter) of mycelium from each strain was inoculated onto PDA plates containing 0.5% Tannic acid as substrate, and the plates were incubated at 25 ºC for 7 days. The formation of a reddish-brown oxidation halo around the mycelia indicates positive laccase secretion (Vantamuri and Kaliwal, 2015). Morphological characterization of potential laccase isolates was done by microscopic examination of lactophenol cotton blue (LPCB) stained smear.

A disc (6 mm) of fungal mycelium from the edge of the colonies culture was aseptically added to 250 mL Erlenmeyer flasks containing 50 mL of autoclaved Potato Dextrose Broth (PDB) medium supplemented with different dye concentrations (250, 500, 1000, 1500 mg L^{-1}). The flasks were incubated using a shaker at 75 rpm at 28 ± 2 ºC for 7 days. Triplicate sets of flasks were used for each fungal species (Hefnawy et al., 2017). The uninoculated medium supplemented with the same dye concentration was used as a control. The supernatant was analyzed for decolorization assay by measuring the absorbance of the supernatant by UV-Vis spectrophotometry. The decolorization activity is expressed as the color reduction percentage (%) and calculated according to Casieri et al. (2008) as follows:

$$\% \text{ Decolorization} = \frac{\text{FA} - \text{LA}}{\text{FA}} \text{ x } 100\%$$

where:

FA = first absorbance
LA = last absorbance

A disc (6 mm) cutting from the edge of the cultivated medium of fungi potential was aseptically inoculated onto a 250 Erlenmeyer flask containing 50 mL of PDB medium supplemented with different concentrations of RBB dye (250, 500, 1000, 1500 mg L^{-1}) as a sole carbon source. The flasks were incubated using a shaker at 75 rpm at 28 ± 2 °C for 5 days. Triplicate sets of flasks were used for each fungus isolate and dye concentration. The supernatant was analyzed for decolorization percentage by measuring the absorbance of the supernatant by UV-Vis spectrophotometry. The percentage decolorization was calculated as described above.

The most potential fungal pellet isolates formed after 3 x 24 hours incubation time, then aseptically separated from the PDB medium (Dewi et al., 2018). The pellets were added aseptically to the 100 mL batik waste that had been sterilized, then incubated using a shaker at 75 rpm at 28±2 ºC for 5 days. One millilitre (1.0 mL) was withdrawn every 24 h and centrifuge at 4000 rpm for 5 minutes. Percentage decolorization was calculated as described above (Ademakinwa and Agboola, 2015).

Two plugs growth of the fungus was grown in a minimal medium and incubated at 32 °C for 10 days (Bhamare et al., 2018). About 1 mL aliquots of the culture medium were withdrawn daily at regular intervals of 24 h and centrifuged at 10,000 rpm for 10 minutes at 4 °C (Eggert et al., 1996). Laccase (Lac) activity was measured by monitoring the oxidation of 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) at 420 nm of reaction mixture consisting of 1.5 mL crude enzyme, 1.5 mL of 100 mM sodium acetate buffer, 1.5 mL of 0.5 mM ABTS incubated at

25 ºC (Bhamare et al., 2018). One unit of the enzyme was defined as the amount of enzyme that oxidizes 1 µM of ABTS per minute at 25 ºC. Laccase activity measured at 420 nm was calculated by using the following formula:

$$U/mL = \frac{\Delta A\ Min^{-1} x\ V}{v\ x\ \varepsilon\ x\ d}$$

where:

V = Total reaction volume (mL)
V = Enzyme volume (mL)
ε = Extinction coefficient of ABTS at 420 nm (ε mM) = $3.6\ x10^4 M^1 cm^{-1}$
d = Light path of cuvette (cm)
$\Delta A\ min^{-1}$ = Absorbance change per minute at 420 nm.

About 200 mg of mycelium formed after being incubated at 28 °C for 72 h was transferred to a sterilized 1.5 mL Eppendorf microcentrifuge tube with a sterilized scalpel (Gontia- Mishra et al., 2014). The mycelium was used for the first step of DNA extraction by following a protocol based on ZR Fungal DNA Kit™ Catalog No. D6005 by ZYMO. Molecular identification was conducted based on amplification and sequencing of the Internal Transcribed Spacer (ITS) region. The ITS region was amplified in a polymerase chain reaction (PCR) using ITS primers of ITS1 (5'-TCCGTAGGTGAACCTGCGG-3' and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Singha et al., 2016). The amplification of DNA was conducted by PCR using MyTaq HS Red Mix that started by preparing the PCR master mix (50 µL), up to 50 µl ddH_2O, 25 µL MyTaqHS Red Mix, 20 µM of each primer, and 200 ng DNA template (Meridian Bioscience). The following cycling condition was used for PCR reaction: Initial denaturation at 95 °C for 1 min, then 35 cycles of 95 °C for 15 s, 57.6 °C for 15 s, 72 °C for 10 s, and 75 °C for 5 min. The product of PCR was stored at 4°C. About 1 µL of PCR products were visualized by electrophoresis with 1% TBE agarose gel to check the quality of the PCR products. Electrophoresis was done for 30 minutes at 100 Volt and then visualized with a UV transilluminator (Wirya et al., 2020).

The DNA sequence was analyzed by alignment using GeneStudio software. The alignment sequence was then used to determine the level of homology by compared against the GenBank database using the NCBI BLAST program. Sequences were then compared with ITS sequence in the GenBank database using BLAST (Salem et al., 2019).

The decolorization activity and dry weight were analyzed using analysis of variance (ANOVA) by using Duncan's test of the data to complete the significant difference test using SPSS 16 software at a 0.05 significance level. All experiments were conducted in triplicate, and the results were expressed as mean ± standard error.

Results

Isolation and qualitative screening of potential laccase-producing fungi

Ninety-eight fungal isolates were obtained from soil, wastewater, and sludge sample cultures. Physicochemical characteristics of batik, including pH, biological oxygen demand (BOD), chemical oxygen demand (COD), total dissolved solids (TDS), and total suspended solids (TSS), were estimated before and after treatment for analysis. The results showed that the batik effluent before any treatment had a 3205.39 mg L^{-1} BOD value, pH of 10.7, along with COD, TDS, and TSS values of 541.72 mg L^{-1}, 5230 mg L^{-1}, and 22.2 mg L^{-1}, respectively. A preliminary screening was performed using tannic acid in an agar medium, which had been used to identify fungi-produced laccase for ninety-eight fungal isolates. Among the 98 isolates, the formation of brown oxidation was developed around six fungal colonies shown in Figure 1 and Table 1, namely 74BRT, 105PDL, 31GYT, 39 GYT, 13GYT, and 2GYA. These indicated a positive reaction of fungal colonies in producing laccase enzymes. While the isolated fungi that did not show a brown zone could be due to not having laccase enzyme activity, so further testing was not carried out.

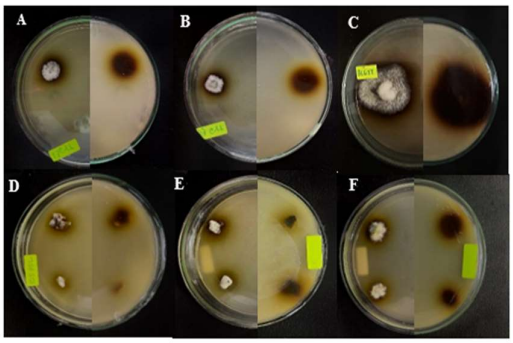

Figure 1. Six selected isolates for secretion of laccase enzyme using tannic acid as substrate. (A) 13GYT, (B) 39GYT, (C) 31GYT, (D) 105PDL, (E) 74BRT, (F) 2GYA.

Quantitative screening for fungal isolates

Figure 2 shows the decolorization of six potential fungal isolates. The best decolorization value of RBB dye for 74BRT and 105PDL isolates was obtained at the initial dye concentration of 250 ppm (96.89% and 91.21%), and it decreased above this concentration which may be due to the dye toxicity. The maximum color removal of the RBB dyes was decolorized to a greater extent by 74BRT and 105PL than other fungi. As can be seen in Figure 3, the maximum color

removal of RBB dyes by the most potential fungal isolates was at 250 ppm of dye concentrations at 120 h incubations.

Morphology of the potential fungal isolates

The morphological characteristics of laccase produced by the most potential fungal isolates were observed. Isolates 74BRT and 105PDL were identified based on their morphological characteristics as *Aspergillus* sp.1 and *Aspergillus* sp. 2 (Figure 4). The isolates identified with the genus *Aspergillus* sp. had macroscopic characteristics of yellow and brownish yellow colonies surface and brownish white colonies on the reverse. The surface of the colonies was rough and granular.

Table 1. Qualitative screening of fungal isolates based on laccase activity.

No.	Sample Code	Collection Sample	Tannic Acid*
1	74BRT	Soil	++++
2	105PDL	Sludge	++
3	31GYT	Soil	++++
4	39GYT	Soil	++++
5	13GYT	Soil	++++
6	2GYA	Effluent	+++

*Scoring: +/- = positive but barely noticeable; + = light to fairly pale color; ++ = moderately pale to strong color; +++ = moderately strong to intense color; ++++ = very intense color (Yadav et al., 2019).

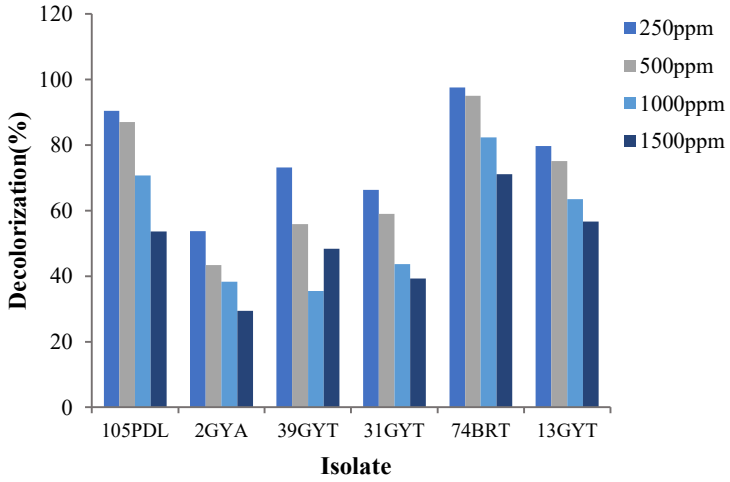

Figure 2. Quantitative screening of Remazol Black B (RBB) dye with various concentrations using six isolates of selected fungi from the isolation.

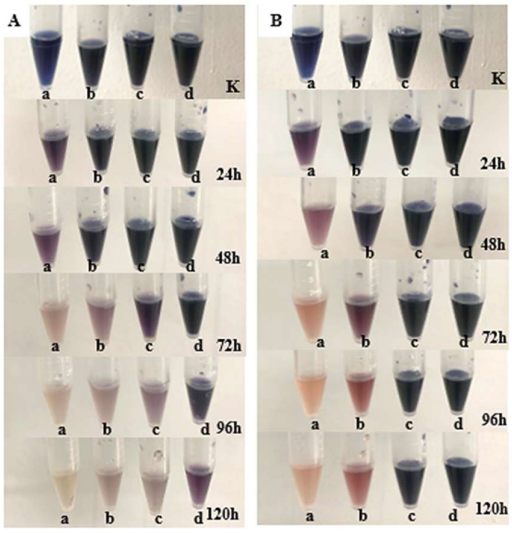

Figure 3. Visual observation of decolorization of Remazol Black B (RBB) dye by two potential fungal isolates. (A) 74BRT, (B)105PDL. Control (K), 250 ppm (a), 500 ppm (b), 1000 ppm (c), and 1500 ppm (d).

The granular color on the surface of the isolated colonies was yellow and brownish. The two isolates had no concentric circles and no exudate drop. The microscopic characteristics obtained were having septate hyphae and long biseriate conidiophores. The ends of the conidiophores from rounded vesicles and on the top layer, there is a metula that produces conidia. The shape of the head of the conidia is the radiate type, with a round to elliptical cell wall structure.

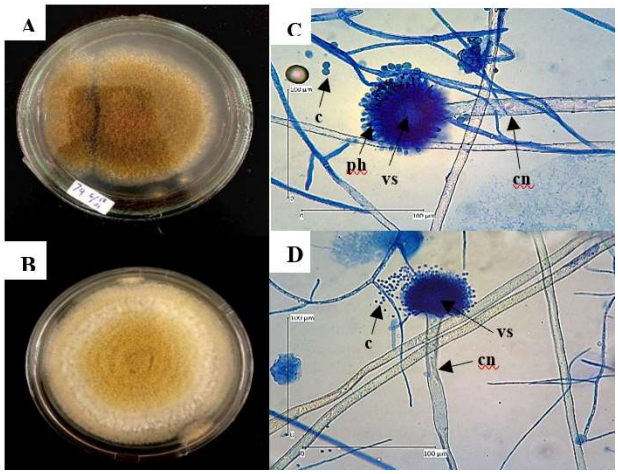

Figure 4. Macroscopic and microscopic morphology (scale bars = 100 µm) of potential fungal isolate grown on Potato Dextrose Agar (PDA) medium after 168 hours. A: Isolate 74; *Aspergillus* sp.1, B: Isolate 105 PDL; *Aspergillus* sp.2, C-D: microscopic morphology of *Aspergillus* sp.1 and *Aspergillus* sp.2 showed conidia (c), conidiophore (cn), phialide (ph), vesicle (vs).

Molecular identification of the potential fungal isolates

The sequences were analyzed by BLAST. Sequencing results of potential fungal isolates showed that the isolate sequence of *Aspergillus* sp.1 had an identity of 100% against *Aspergillus tamarii* (MH345894.1), while the isolate of *Aspergillus* sp.2 had an identity of 100% against *A. sclerotiorum* (KT581403.1). *A. tamarii* was the potential isolate in this study because it had the best ability to decolorize Remazol Black B (RBB) dye up to 97.53%. Using sequence analysis of the ITS region of the ribosomal RNA gene cluster, we investigated the genetic relatedness of section *Aspergillus* isolates (Figure 5). With a bootstrap value of 90% phylogenetic analysis of ITS gene data revealed that *Aspergillus* sp. 1 was closely related to *Aspergillus tamarii 54* (MH345894.1) but phylogenetically distinct from *Aspergillus sclerotiorum WSMT12, Aspergillus niger OZ-3, Aspergillus ochraceus ANDD01 and Aspergillus fumigatus ATCC 1022. Aspergillus* sp.1 clade contained species with conidia in shades of olive to brown, and this clade includes *Aspergillus tamarii* 54.

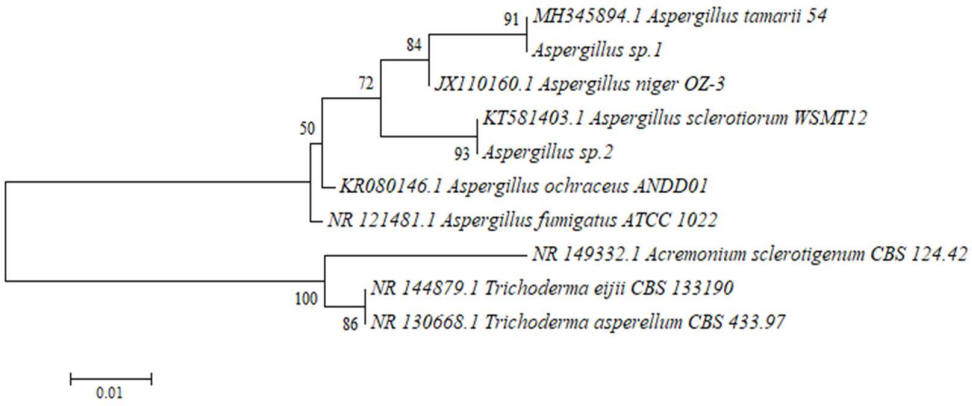

Figure 5. Molecular phylogenetic analysis of *Aspergillus* sp.1 and *Aspergillus* sp.2. based on the Neighbour-Joining method, the numbers at the nodes represent the levels of bootstrap value.

While *Aspergillus* sp.2 was closely related to *Aspergillus sclerotiorum WSMT12* (KT581403.1) with the bootstrap value of 93%, but phylogenetically was distinct from *Aspergillus tamarii 54, Aspergillus niger OZ-3, Aspergillus ochraceus ANDD01* and *Aspergillus fumigatus ATCC 1022. Trichoderma asperellum CBS 433.97, Trichoderma eijii CBS 133190,* and *Acremonium sclerotigenum CBS 124.42* was the outgroup.

Decolorization assay of the potential fungi based on various concentrations of Remazol Black B dye and incubation time

Table 3 shows that decolorization activity for both fungi was optimum at 120 hours of incubation, with 97.87% and 93.63% for *Aspergillus tamarii* and *A. sclerotiorum,* respectively, at an initial dye concentration of 250 mg L^{-1}. The best dye decolorization occurred by *A. tamarii* at a concentration of 250 mg L^{-1} and exhibited a 97.87% decolorization value of RBB dye.

During the decolorization process, the incubation time of 120 hours showed a change in the pH value of the RBB dye by *A. tamarii* and *A. sclerotiorum* (Table 3). The pH value at each concentration generally changes to 6-7.

Decolorization assay of the batik effluent by the potential fungal isolates

The two selected fungal isolates *Aspergillus tamarii* and *A. sclerotiorum* were tested for their decolorization ability against batik mixed waste of various batik dyes that have been used by the batik industry. Figure 6 showed that *A sclerotiorum* exhibited the highest decolorization activity of batik waste at 42.09%. Meanwhile, *A. tamarii* could decolorize batik waste by 37.47%.

Table 3. Effect of various concentrations of Remazol Black B dye and incubation time on decolorization percentage of *Aspergillus tamarii* and *Aspergillus sclerotiorum* grown on Potato Dextrose Broth medium at 28 ºC for 5 days.

Concentration (mg L^{-1})	Incubation time	Decolorization (%)			
		*Aspergillus tamarii**	pH	*Aspergillus sclerotiorum*	pH
250	24	51.43 ± 0.11^{hi}	4.72	51.20 ± 0.11^{fghi}	4.83
	72	87.61 ± 0.03^{c}	5.85	81.44 ± 0.04^{b}	5.25
	120	97.87 ± 0.00^{a}	6.55	93.62 ± 0.01^{a}	5.70
500	24	35.65 ± 0.08^{ijkl}	7.22	40.56 ± 0.10^{hi}	7.90
	72	76.81 ± 0.03^{def}	5.93	70.24 ± 0.03^{d}	5.52
	120	93.18 ± 0.02^{b}	6.40	85.94 ± 0.01^{b}	6.10
1000	24	26.22 ± 0.08^{kl}	7.33	25.18 ± 0.05^{jk}	8.21
	72	59.94 ± 0.05^{h}	6.37	57.26 ± 0.01^{efg}	7.45
	120	81.18 ± 0.04^{de}	6.24	71.30 ± 0.01^{cd}	7
1500	24	16.97 ± 0.06^{l}	8.54	11.44 ± 0.05^{k}	7.68
	72	32.37 ± 0.08^{jkl}	8.30	41.91 ± 0.06^{ghi}	7.27
	120	63.65 ± 0.07^{gh}	7.04	52.64 ± 0.04^{efgh}	6.20

*Numbers followed by different letters show a significant difference at 5% probability, according to Duncan Multiple Range Test (DMRT).

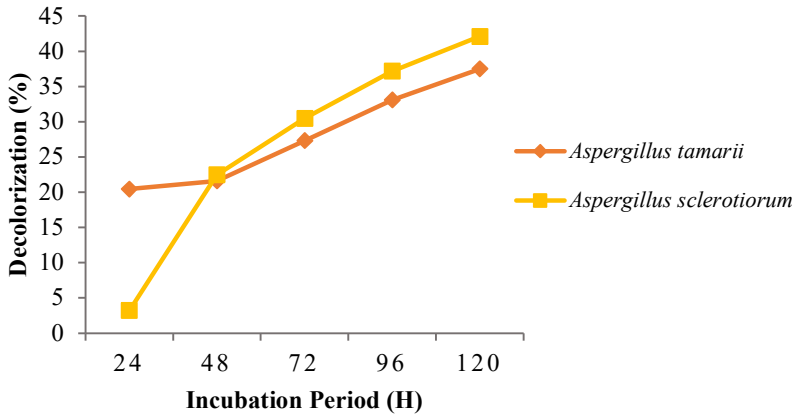

Figure 6. Decolorization of batik effluent by *Aspergillus tamarii* and *Aspergillus sclerotiorum* at 28 ± 2 ºC for 5 days.

Laccase activity

The effect of the incubation period on laccase production by *Aspergillus tamarii* and *A. sclerotiorum* was shown in Figure 7. The gradual increase in time led to a concomitant increase in the amount of laccase. Optimum incubation was found to be 7 days, where 12.23 IU mL^{-1} and 9.34 IU mL^{-1} were assayed. Enzyme production increases with time till 7 days. More increase in incubation period above this optimum led to a decrease in enzyme activity.

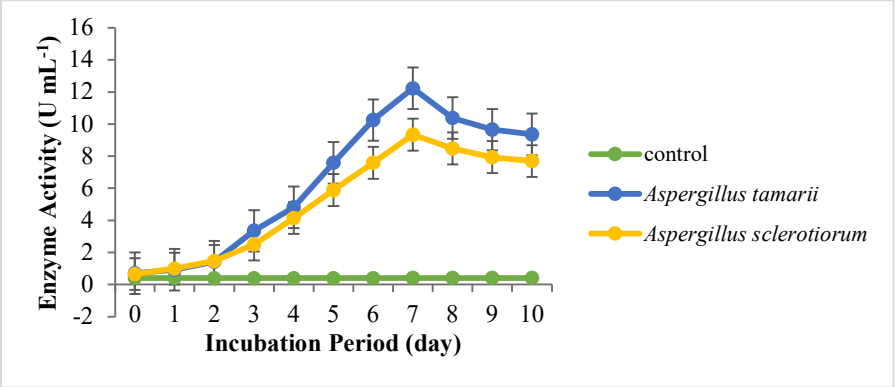

Figure 7. Laccase activity by *Aspergillus tamarii* and *Aspergillus sclerotiorum* at 28 ± 2 ºC for 10 days incubation.

Discussion

After three days of incubation, the oxidation of tannic acid was visually scored for laccase activity as a preliminary screening for laccase production (Yadav et al., 2021) (Table 1). About six fungal colonies had a positive reaction in producing laccase enzymes which showed a brown zone around their colonies. While the isolate that did not show a brown zone could be due to not having a laccase enzyme activity. These were in line with the research conducted by Vantamuri and Kaliwal (2015), where the results of the laccase test using tannic acid only produced eight potential isolates from 150 isolates of fungi. Six fungal isolates in this study were selected for further study.

Synthetic dyes are increasingly employed in textile dyeing and finishing processes due to their ease of use, low cost, stability, and large color palette (Sadhasivam et al., 2009). When thrown improperly in an aquatic environment, synthetic dyes have a complex aromatic structure that makes them resistant to light, ozone, and other degradative environmental factors. Remazol Black B (RBB) is an azo-type dye that, under anaerobic conditions, can be degraded into aromatic amines which have carcinogenic potential (Leal et al., 2021). The high decolorization efficiency of 74BRT and 105PDL isolates at an initial dye concentration of 250 mg L^{-1} was a greater result than the observation obtained by Hefnawy et al. (2017); they reported that *Aspergillus flavus* and *Penicillium canescens* had the maximum decolorization of direct blue dye at the initial concentration of 100 mg L^{-1}, and it decreased above this concentration. Fetyan et al. (2016) also reported that *Saccharomyces cerevisiae* could decolorize direct blue dye 71 up to 100%, but the percentage of decolorization decreased above this concentration. The maximum color removal of the RBB dye was decolorized to a greater extent by 74BRT and 105PDL than other fungi. It means that 74BRT and 105PDL had the best criteria for potential fungi based on qualitative and quantitative screening and were selected for further study.

Based on the morphological characteristics, 74BRT and 105PDL isolates were identified as *Aspergillus* sp.1 and *Aspergillus* sp.2. The isolates belonging to the genus *Aspergillus* characteristically present dark-brown to black conidia, with uniseriate or biseriate conidiophores, spherical vesicles, and hyaline or lightly pigmented hyphae near the apex (Silva et al., 2011). *Aspergillus* sp.1 and *Aspergillus* sp.2 had yellow-dark brown conidia globose with 5-8 µm in diameter, biseriate conidiophores, spherical vesicles varying greatly in size from 20-50 µm, and hyaline pigmented hyphae near the apex.

Molecular identification based on the ITS rDNA showed that *Aspergillus* sp. 1 had 100% identity with *Aspergillus tamarii* 54 (MH345894.1). *Aspergillus tamarii* was reported by Saraswathy et al. (2010) could decolorize Coomassie Brilliant Blue (CBB), Bromophenol Blue (BPB), and Malachite Green (MG) dyes up to > 90%. The BLAST result of ITS rDNA sequence of *Aspergillus* sp.2 showed 100% identity with *Aspergillus sclerotiorum* (KT581403.1). *Aspergillus* sp. 3 was reported by Dewi et al. (2019) could decolorize Indigosol Blue O4B up to 96.6%. Da Silva et al. (2018) reported *A. sclerotiorum* CBMAI 849 is an efficient fungus for decoloration of Remazol Brilliant Blue R (RBBR) by 95%.

The dye decolorization percentage was decreased by increasing dye concentrations on RBB dye by *Aspergillus tamarii* and *Aspergillus*

sclerotiorum. These might be caused by the structure of the compounds of dye which are more complex and cause alkaline conditions (Zhu et al., 2005). Table 3 showed that at higher the concentration of the dye, the higher the pH value. According to Legorreta-Castaneda et al. (2020), biosorption occurs when a pollutant interacts with active groups on the surface of fungal cells, such as chitins, acid polysaccharides, lipids, and amino acids. Receptors on the surface of fungal cells increase the ionization process when the pH is acidic, so the binding process of complex compounds of the dye might be increased. Sathiya et al. (2007) reported that the decolorization process of *Aspergillus niger* at pH 11 might be caused by the particles of dye taking longer for absorbed onto the cells, which makes the rate of decolorization decreased. These might be the reason which makes the rate of decolorization percentage decrease.

The effect of the incubation period on decolorization activity showed that *Aspergillus tamarii* and *A. sclerotiorum* had the maximum decolorization activity at 120 hours of incubation. Purnama and Setiati (2004) stated that these might be caused by the interaction between the sorbent and the dye, where the longer the incubation time, the more dye absorbed. The results of this study are similar to Wulandari et al. (2014), who showed that *Pleurotus ostreatus* treated with different incubation times for decolorizing batik waste showed a percentage value of decolorization between 33.05% up to 85.64% along with longer of incubation time. In a study conducted by Ning et al. (2017), *A. flavus* and *Populus canescens* reached the maximum value of decolorization at 168 hours of incubation, where it exhibited 97% and 92% for both fungal isolates. According to Hefnawy et al. (2017), until the 168 hours of the incubation period, *A. flavus* showed an increasing decolorization of Direct Blue dye by 97%. However, after 168 hours of incubation, *A. flavus* shows a decrease in the ability to decolorize the dye. These might be occurred because of the enzymatic biodegradation activity, in which the binding process of dye particles on the fungal biomass and the accumulation of dyes can inhibit the growth and metabolism of fungi due to the decolorization process.

During the decolorization process, up to the incubation time of 120 hours showed a change in the pH value of the RBB dye by *Aspergillus tamarii* and *A. sclerotiorum* (Table 3). The pH value at each concentration generally changes to 6-7. This is in line with research conducted by Yulita et al. (2013) that the decolorization process by *Pleurotus ostreatus* mycelium affects changes in pH. The pH value after treatment ranged from 5.26-6.84. These results indicate that the decrease in pH occurred due to the utilization of batik waste as a substrate by *P.ostreatus*.

When the *A. tamarii* and *A. sclerotiorum* were tested for their decolorization ability against batik effluent, it showed that both fungi had a poor consistency of decolorization ability. The decolorization percentage only reached 37.47% and 42.09%. The low value of decolorization by *A. tamarii* and *A. sclerotiorum* was similar to Ranjitha et al. (2018), in which *A. niger* was able to decolorize Malachite Green dye up to 92.85%, Nigrocin dye up to 93.33%, and Basic Fuchsin dye up to 90.05% which were incubated for 7 days at room temperature. However, when grown into a mixture of the three dyes, *A. niger* ability to decolorize the dyes was decreased. The percentage of decolorization in mixed waste only reached 32.33%.

The mixture of batik dyes in this study made the concentration of the batik waste higher and had a high pH value (10.3) than the concentration of RBB dyes. The high pH value of batik waste can affect the ability of fungi to grow and carry out metabolic processes. These results are similar to those of Abd El-Rahim et al. (2009) that the biosorption rate of the dye became low when the incubation process of the fungal isolate was carried out at pH 10. This was presumably due to a decrease in the accumulation of fungal biomass so that the biomass that was able to grow was not sufficient to decolorize the dye by more than 23%. Increasing the pH in the solution caused a decrease in the decolorization process. In addition, the final waste containing mixtures of dyes had a variety of more complex chromophore structures that make it difficult to degrade, thus becoming one of the factors that affect the percentage of decolorization. This statement could be the reason for the low ability of decolorization of batik waste by *Aspergillus tamarii* and *A. sclerotiorum* in this study.

The batik effluent decreased with removal percentages of 42.10, 37.13, 36.1, and 21.2% for BOD, COD, TSS, and TDS due to the decolorization activity of *A. tamarii*. Meanwhile, the batik effluent was decreased with removal percentages of 37.44, 42, 42, and 31.3% for BOD, COD, TSS, and TDS, respectively, due to the decolorization activity of *A. sclerotiorum.* After the decolorization treatment, the pH value of batik effluent decreased in the range of 7.1-8.7 by *A. tamarii* and *A. sclerotiorum.* The decrease in BOD and COD levels by *Aspergillus tamarii* and *A. sclerotiorum* tends to be low (<50%). However, the decrease in BOD and COD values that occurred can explain that the amount of organic matter contained in the batik effluent had been partially decomposed (Zaoyan et al., 1992). The high value of TDS in this study can interfere with the diffusion of sunlight into the water, causing the photosynthesis process by aquatic flora to be disrupted. If this happens, it can cause a reduction in dissolved oxygen levels in water (Namdhari et al., 2012).

Fungi have created enzymes for the decolorization of azo dyes, and dye molecules have wide structural variability, with just a few enzymes capable of degrading them. Azo reductases and laccases appear to be the most promising enzymes for

azo dye enzymatic cleanup. According to Wong and Yu (1999), these enzymes are multicopper phenol oxidases that decolorize azo dyes by generating phenolic compounds by a very nonspecific free radical process, avoiding the creation of harmful aromatic amines.

This study found that *Aspergillus tamarii* and A. *sclerotiorum* presented a high capacity for decolorizing RBB dye. The optimum incubation for laccase assay was found to be seven days, where 12.23 IU mL^{-1} and 9.34 IU mL^{-1} were assayed. The presence of enzyme activity in *A. tamarii* and *A. sclerotiorum* could be the reason for their high ability to decolorize RBB dye. It is because the activity of the laccase enzyme could be stimulated by the presence of an inducer. In this study, the analysis of laccase enzyme activity used a CuSO4 inducer which is a phenolic group. However, according to Bertrand et al. (2013), the laccase enzyme could be induced by several inducers, such as aromatic compounds or compounds that had a structure similar to lignin. Murugesan et al. (2007) stated that Remazol dye had aromatic compounds, so the presence of Remazol dye can increase the activity of the laccase enzyme. This indicated that the high activity laccase enzyme of *A. tamarii* and *A. sclerotiorum* was in line with their high ability to decolorize RBB dye up to >90% (97.87% and 93.62%).

Optimum incubation was found to be seven days, where 12.23 IU mL^{-1} and 9.34 IU mL^{-1} were assayed. Enzyme production increases with time till seven days. More increase in incubation period above this optimum led to a decrease in enzyme activity. The decrease in enzyme synthesis could be attributed to nutrient depletion in the production medium (Abd El Monssef et al., 2016). In an earlier study, maximum laccase production by *Aspergillus flavus* was also obtained on the 7th day of incubation. According to Aftab et al. (2018). *A. flavus* Maf 0139 sharp increase in the production of laccase was observed from the 5th to 7th-day incubation. Further increase in the time incubation did not enhance enzyme production and resulted in a significant decrease after 15 days of incubation.

Conclusion

Aspergillus tamarii and *A. sclerotiorum* were capable of decolorizing Remazol Black B dye with a high percentage of 97.87% and 93.62% after 120 hours of incubation. Both fungi *A. tamarii* and *A.sclerotiorum* also had the ability to decolorize the batik effluent at 37.47% and 42.09%, respectively.

Acknowledgements

This research was financially funded by 'Rekognisi Tugas Akhir (RTA) Grant 2022' (ST No. 3550/UN1.P.III/Dit-Lit/PT.01.05/2022) from Universitas Gadjah Mada (UGM), Indonesia. The authors thank members of Plant Systematics Laboratory and Falitma Laboratory Faculty of Biology Universitas Gadjah Mada. This article is part of Y.S. Ayu thesis under the supervision of R.S. Kasiamdari.

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